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TESI DI DOTTORATO DI RICERCA

SELF-AMPLIFYING RNA VECTORS ENCODING SURVIVIN AS ANTI-TUMORAL VACCINE CANDIDATES

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Ai nonni Gianni ed Antonio,
sempre al mio fianco in questi anni

Table of Contents

1. INTRODUCTION

1.1 Cancer and immunotherapy

1.1.1 Cancer treatments

Cancer is the second-leading cause of death in humans, it is a group of diseases characterized by an abnormal growth of cells, which tend to proliferate in an uncontrolled manner. Cancer can involve any tissue of the body and it is able to invade nearby tissues and to spread to more distant sites through blood or lymphatic vessels, causing metastases. The hallmarks of cancer cells comprise six biological capabilities which tumor acquires during its multistep development: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan et al., 2011). The feature of tumor which joins all its hallmarks is the genetic instability resulting in the expression of aberrant or elevated levels of a variety of cellular proteins, which can be released in the bloodstream or remain on the cell surface. These proteins are called tumor associated antigens (TAAs) and can also be shared by different tumors.

In addition to cancer cells, a repertoire of recruited, ostensibly normal cells contribute to the acquisition of hallmarks traits by the creation of the "tumor microenvironment".

Many therapies have been developed to treat cancer: surgery, chemotherapy, radiation therapy, and immunotherapy. Surgery is the method of choice for solid cancer treatment, but sometimes it cannot assure elimination of all cancer cells. Chemotherapy, based on cytotoxic anti-neoplastic drugs, is usually combined with surgery, but this therapy can also damage healthy cells as a side effect. Radiation therapy uses ionizing radiation to kill cancer cells, and this therapy is typically used in synergy with surgery or chemotherapy, but the treatment can also affect some of the normal nearby cells causing for example sore skin. Moreover, many tumors are resistent to radiation and chemotherapy. Despite great therapeutic progresses, cancer is still a major public health problem worldwide, requiring new strategies and treatment modalities to optimize patient prognosis. In this context, immunotherapy has always been an attractive and potentially efficient treatment for cancer patients, often in combination with traditional therapies.

Cancer immunotherapy aims at harnessing and enhancing the immune system to specifically target tumor cells and kill them (Aguilar et al., 2011). TAAs are potential targets of the immunotherapy because they are highly expressed by many tumors, but poorly or not at all by normal cells.

1.1.2 Cancer and immune system

Immunity results from the interplay between the innate and the adaptive immune systems. The innate immune system is antigen-nonspecific, based on non-clonal recognition receptors, such as lectin and Toll-like receptors (TLRs). The adaptive immune system, which in contrast is antigen-specific, is based on the capability of B and T cells to use clonal receptors to identify antigens, or their derived peptides, in a highly specific manner. Antigen presenting cells (APCs), such as dendritic cells (belonging to innate immune system), represent an essential link between the two systems. APCs are able to present antigen to CD4+ T cells and CD8+ T cells, activating the adaptive immune response. In normal conditions, the immune system has the natural capacity to detect and destroy abnormal cells thus preventing cancer, but cancer cells sometimes become able to avoid detection and destruction. Cancer cells in fact can activate various escape mechanisms including a reduced expression of tumor antigens, making them harder to be detected, or expression of certain proteins on the cell surface or the release of soluble factors in the cancer microenvironment which suppress the immune response and promote tumor progression (Palucka et al., 2012).

The relationship between the immune system and cancer is complex, in fact the immunogenicity of a tumor is influenced by the immunological enviroinment. The immune system either can block tumor growth, development and survivial or can facilitate tumor outgrowth. This immune system feature is called cancer-immunoediting. The cancer-immunoediting consists of three phases: elimination, equilibrum and escpe. The elimination phase (known as cancer immunosurveillance) consists of the recognition and killing of transformed cells by the innate and the adaptive immmune system. This mechanism of recognition leads to direct killing of tumor cells, in addiction to chemokine and other cytokines released to facilitate tumor cells death, with the aim of eradicate the developing of tumor, protecting the host from cancer formation (Buonaguro et al., 2011; Mellman et al., 2011))

If some cancer cells are not killed during the first phase, the process progress to the equilibrium phase, in which the tumor persist but there is a balance between the immune response and the cancer cells. This balance can tilt towards tumor growth, as a result of immune inhibition or exhaustion or after the emergence of tumor-cell variants, and tumor become able to evade immune pressure. The evasion phase concludes with the apparence of clinically detectable, progressively growing tumors (Dunn et al., 2006).

Another aspect that modulate the immune response is the presence of regulatory T cells (Treg), which account for 5% to 10% of peripheral CD4+ T cells (Gavin et al., 2003), whose key role is to inhibit self-reactive effector T cells, inducing peripheral T cell tolerance (Sakagushi et al., 2005). Treg cells have been found to be increased in peripheral blood and tumors in many human cancers (Ichihara et al., 2003) resulting in poorer prognosis and reduced survival (Yuan et al., 2010)

1.1.3 Cancer vaccines

Cancer immunotherapy may be classified into passive or active therapies. Passive immunotherapy is based on the administration of antitumor antibodies or transfer of tumor-reactive lymphocytes. Active immunotherapy aims to elicit a specific immune responce against tumor associated antigens by the administration of cancer vaccines or to amplify an existing antitumor immune response by employing proinflammatory molecules or adjuvants (Buonaguro et al., 2011).

Cancer vaccines are designed to induce the immune system to recognize and attack cancer cells with the aim to stop further growth of cancer, prevent the cancer to coming back and destroy any cancer cells in the body. Several types of cancer vaccine have been developed, such as whole cell-, dendritic cell-, or DNA based-vaccines. In particular, vaccines based on viral vectors represent a strategy which has been studies for a long time. Vector-based cancer vaccine strategies consist in the injection of a vector encoding a TAA in the muscle, where myocytes and muscle-resident APCs can be transfected by the vaccine. In transfected myocytes the antigen expressed inside the cell is loaded on MHC-class I, and subsequently presented to APCs. APCs which are directly transfected also present antigen peptides in the context of class I MHC molecules. Conversely, APCs exposed to antigen producing myocytes, or other cells, uptake the antigen and present it, as peptides, within the context of MHC class II. Finally, APCs presenting antigens in MHC class I and/or class II

move to lymph nodes where they can activate CD8+ T cell and CD4+ T cells, respectively, which develop the ability to target tumor antigen and kill cancer cells (Fig.1).

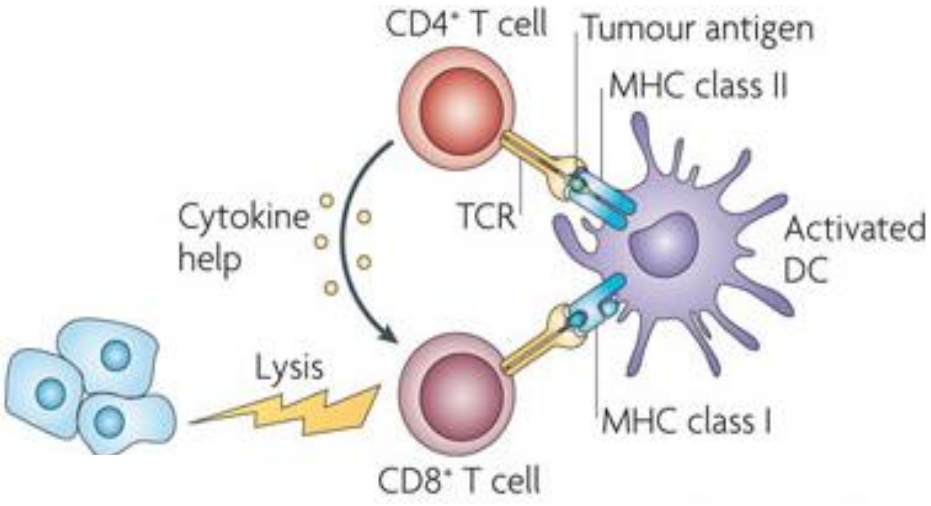

Fig.1 Vaccine-based immunotherapy
Vaccine encoding tumor antigen can be uptaken by dendritic cells (DCs), which expressed the antigen on MHC class I or II. Antigen presentation activates CD4+ T cells and CD8+ T cells, which reach tumor and lyse cancer cells.

Two kinds of cancer vaccines exist: one is the preventive vaccine, which avoids tumor development and may be the only cancer treatment with the potential to last for a lifetime (an example is the human papillomavirus (HPV) vaccine for cervical cancer prevention). The other is the therapeutic vaccine, which helps the immune system to fight cancer by amplifying its capacities to recognize and kill cancer cells. Treated subjects can mount an immune response able to either cure tumor or keep it under constant restraint, delaying tumor recurrence and prolonging survival (Vergati et al., 2010).

The major difficulties in developing an efficient cancer vaccine are the paucity of TAAs (expressed only by tumor cells) and the weakness of the immune response against tumor antigens, because they are often recognized as self-antigenes and therefore protected by immune tolerance. Various strategies for therapeutic cancer vaccines have been proposed to enhance this weak immune

response, including vaccines based on cells, DNA, RNA, protein, peptides, and viral vectors (Vergati et al., 2010).

1.1.3.1 Recombinant poxvirus vaccine

Viral vectors represent one of the widely employed strategies and are regarded as the most efficient means to deliver nucleic acids and to induce the immune response with high efficiency. Viral vector vaccination is expected to induce appropriate "danger" signals to the immune system resulting in a preferential recognition and presentation of the target antigens. Among several viral vectors available, Poxviruses have been considered as valuable tools for cancer vaccine development. The Poxviridae family is divided in a number of subfamilies, based on their host range. Poxviruses are enveloped dsDNA viruses that replicate uniquely in the cytoplasm of infected vertebrate or invertebrate cells (Moss, 2007). This life cycle confers certain advantages as vaccine delivery system, because the potential for latent infection or insertional mutagenesis is eliminated (Larocca at al., 2011).

Poxvirus-based vaccines have been widely used for long time in infectious disease treatment, such as the smallpox vaccine, developed by Edward Jenner in the 18^{th} century that eradicated the disease in 1979, and also in AIDS treatment (Gomez et al., 2012). In the last decades poxvirus-based vectors have also been tested also as tumor vaccines by expressing recombinant TAA transgenes, showing positive results. Poxvirus-based vaccines present several features that make them suitable for vaccinations: they can be genetically manipulated to express transgenes with high stability providing potent gene delivery; they can harbour large amount of DNA and are capable to induce both humoral (antibodies) and cytotoxic T cell responses against the encoded antigen, with long lasting immunity (Pastoret and Vanderplasschen, 2003).

Over the past ten years, about 30 clinical trials tested therapeutic vaccinations with poxvirus-based vectors expressing different tumor antigens: CEA (carcinoembryonic antigen) (Marshall et al., 2000), PSA (prostate specific antigen) (Kaufman et al., 2004), melanoma antigens (gp100, MART-1/Melan-1, tyrosinase) (Zajac et al., 2002) and human papilloma virus proteins E2, E6 and E7 (Corona Gutierrez et al., 2002). Table 1 summarizes some of the poxviral vaccines in clinical development (www.clinicaltrials.gov).

Virus	*Gene*	*Disease*	*Cytokines*
Vaccinia	CEA	Colorectal cancer	
	PSA	Prostate cancer	
	MUC-1	Pancreatic cancer	GM-CSF
	gp100	Melanoma	IL-2
	MART-1/Melan-A	Melanoma	IL-2
	Tyrosinase	Melanoma	IL-2
	HPV E6 and E7	Cervical cancer	
	B7.1	Melanoma	
	TRICOM*	Melanoma	
	CEA-TRICOM	Colorectal and pancreatic cancer	GM-CSF, IL-2
	PSA-TRICOM	Prostate cancer	GM-CSF
	GM-CSF	Bladder cancer	
Modified Vaccinia Ankara	5T4	Colon, breast, and renal carcinoma	IL-2
	HPV E2	Cervical cancer	
Fowlpox	CEA	Colorectal cancer	GM-CSF
	PSA	Prostate cancer	
	gp 100	Melanoma	IL-2
	Tyrosinase	Melanoma	IL-2
	B7.1	Melanoma	
	TRICOM	Melanoma	
	CEA-TRICOM	Colorectal cancer	GM-CSF
	GM-CSF	Colorectal cancer	

Table 1. Poxviral vaccine in clinical development
The table presents examples of Poxvirus-based cancer therapies

Viral vector-based vaccines optimize antigen immunogenicity, but relevant limitations, such as a pre-existing immunity against the vector can decrease their efficacy (e.g. most aged people have been vaccinated against smallpox). After immunization, they also induce a strong neutralizing antibodies against the vector itself. This reaction reduces the success of a subsequent vaccination performed with a homologous poxvirus vector, decreasing also the expression of the encoded antigen, thus making repeated immunizations with the same vectors inappropriate (Larocca et al. 2011).

1.1.3.2 Self-amplifying RNA vaccines and cell delivery

To overcome viral vector limitations, to provide antigen protection from degradation, and facilitate its entry into cells, other vaccination strategies have been considered: DNA vaccination, protein or peptide-based vaccine, dendritic cells- or RNA based strategy. In particular, in this study a new vaccine technology based on self-amplifying RNA has been studied.

1.1.3.2.1 Self-amplifying RNA vaccines

Self-amplifying RNA vaccines are based on the genome of Alphavirus, which are positive-sense, single-stranded RNA of about 10 kb, containing a 7-methyl-G cap at its 5' terminus and a poly(A)-tail at its 3' terminus. Self-amplifying RNA vaccines contain four genes encoding the non-structural proteins (nsP1-4) that form the RNA replication machinery. The structural protein genes, required to produce infectious particles, have been deleted and replaced with a gene of interest, which is abundantly expressed from a subgenomic mRNA.
RNA is produced *in vitro* by an enzymatic transcription reaction from a linear DNA using a T7 RNA polymerase, thereby avoiding safety concerns and complex manufacturing issues associated with cell cultures. After immunization, replication and amplification of the RNA molecule occurs exclusively in the cytoplasm of transfected cells eliminating risks of genomic integration and cell transformation. As the original virus, upon internalization inside the cells, RNA immediately starts to translate the viral non-structural proteins (encoded at the 5' end of the genome) forming the RNA-dependent RNA polymerase (RDRP), which transcribes the RNA into a negative-sense copy of the genome. From such negative strand, RDRP catalyzes the transcription of a new positive RNA strand, allowing vector amplification, and the transcription of the subgenome with consequent expression of the protein of interest (Geal et al., 2012; Maruggi et al., 2013) (Fig.2).

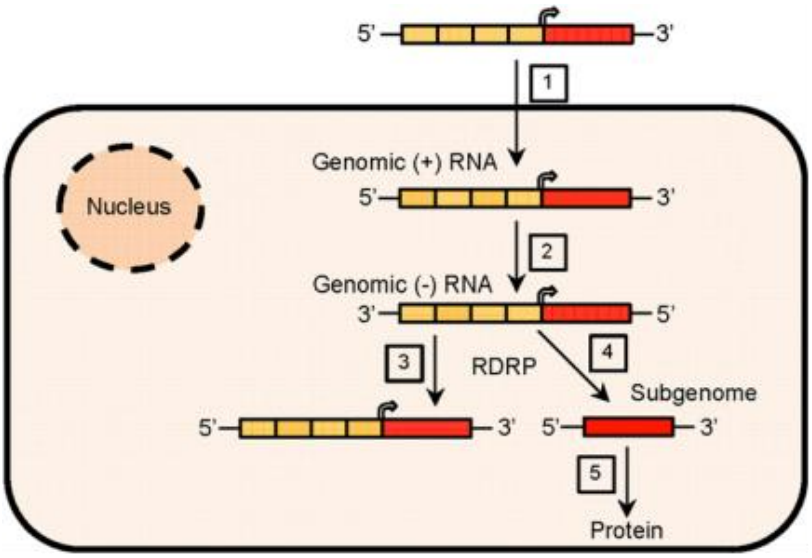

Fig.2 Replication and expression of self-amplifying RNA in a mammalian cell
(1) Delivery of RNA to the cytoplasm. (2) Translation of the ORF encoding the four nonstructural proteins that form the RNA-dependent RNA polymerase (RDRP), which produces a negative-sense copy of the genome. (3) RDRP catalyses production of positive-sense genomes from the negative sense copy. (4) RDRP catalyses transcription of the subgenome. (5) Translation of the gene of interest, leading to protein expression (Geal et al 2012)

Alphavirus-based vaccines have been shown to provide robust antigen expression, due to the fact that the subgenomic RNA is transcribed with an efficiency 3-fold greater than genomic RNA. This high expression of the antigen, with subsequent proteasome processing of peptides, leads to efficient presentation by major histocompatibility complex (MHC) class I; moreover trans-presentation, through MHC class II proteins, also occurs. Thus, the result is a potent antigen-specific immune response (Ljungberg and Liljestrom, 2015). Furthermore, the *in situ* formation of double-stranded RNAs (dsRNAs), produced during RNA replication, stimulates aspects of innate immunity response, such as type I interferon (IFN) production. The activation of innate immune results in heightened the anti-tumor effect (Jin et al., 2010), therefore alphavirus-based vector adjuvates adative immune response (Thompson et al., 2008a).

The alphavirus vector used in this work is a chimeric replicon generated from the genome of the Venezuelan equine encephalitis virus (VEE) truncated of all structural genes and engineered to contain the 3' end untranslated region and the packaging signal of Sindbis (SIN) virus (Fig.3a). The chimeric replicon combines the best features of the two viruses and avoids safety issues. In the generated replicon, the gene(s) of interest replaces capsid and glycoproteins genes, essential for viral particles production. The lacking capsid and E2/E1 glycoproteins are expressed by defective helper RNAs that can be used in combination with the chimeric replicon if viral replicon particles

(VRPs) are to be produced, as in the case of delivery of RNA (Perri et al., 2003; Maruggi et al., 2013) (Fig.3b).

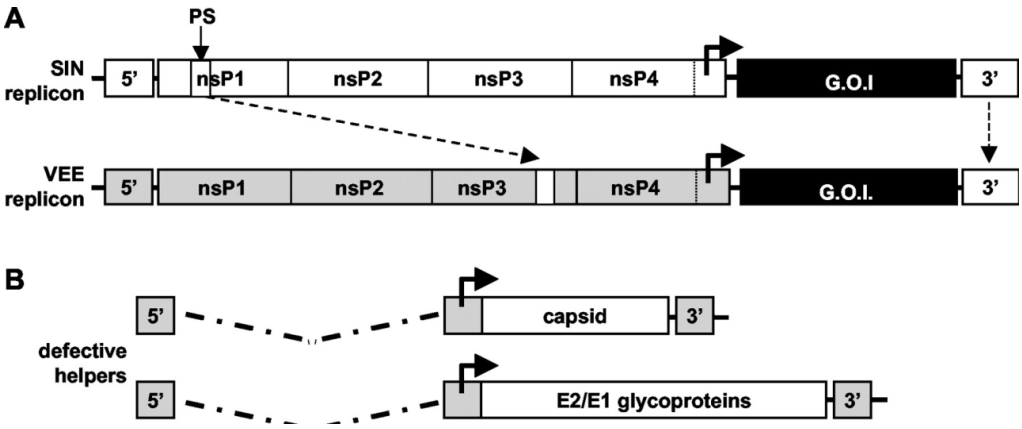

Fig.3 Replicon particle chimeras
(A) Construction of the chimeric replicon. The packaging signal (PS) and 3'UTR (3') from the SIN replicon were used to replace sequences in nsP3 and at the 3' end of the VEE replicon, respectively. (B) VEE-derived defective helpers expressing SIN capsid and envelope glycoproteins. SIN-derived sequences (white boxes), VEE-derived sequences (shaded boxes), and the subgenomic promoter (arrows) are indicated (Perri et al 2003).

Alphavirus replicon based-vaccines have already been employed in clinical trials both in infectious diseases and in cancers treatment, such as in prostatic and colorectal carcinoma therapy (Bernstein et al., 2009; Morse et al., 2010; Slovin et al., 2013), generating both antibody and T cell responses (Bernstein et al., 2009; Morse et al., 2010).

Self-amplifying RNA vector can be delivered inside cells through different strategies, such as viral replicon particles (VRPs), packaging SAM vector in capsid and glycoprotein-made particles, or by nonviral delivery.

1.1.3.2.2 Viral replicon particles (VRPs) delivery system

Viral replicon particles (VRPs) are propagation-defective virus-like particles that allow RNA delivery. Alphavirus capsid and glycoproteins, the structural proteins missing in replicon vectors, are needed for VRPs production. Capsid and glycoproteins are provided by two helper RNAs, lacking the packaging signal for incorporation into the VRPs (Pushko et al., 1997), therefore only if the two helper RNAs are present in a packaging cell line with the replicon encoding the gene of interest, VRPs can be produced (Fig.4). The use of VRPs has been widely documented in literature, and VRPs has been demonstrated to be potent vaccines in mice (Perri S, et al. 2003), nonhuman primates (Barnett SW, et al. 2010), and humans (Bernstein DI, et al. 2009). Being single-cycle particles, VRPs are considered safe because when they deliver RNA inside cells they cannot spread in the host. VRPs have been shown to induce high titers of antibodies and robust antigen-specific T-cell responses in mice (Leitner et al., 2003) and in human subjects (Morse et al., 2010). Of note, VRPs can be delivered to a variety of cell types, including APCs and myocytes , without the need of further formulations, thus they are readily exploitable for mice immunizations and suitable for testing new immunization schedules and/or new experimental set-ups. VRPs protect RNA from degradation allowing the correct delivery of the construct. Compared to viral vectors, VRPs do not present pre-existing immunity against the vector and they are poorly immunogenic, allowing repetitive vaccinations (Uematsu et al., 2012; Durso et al., 2007). Considering all these features, they represent an optimized delivery strategy for RNA.

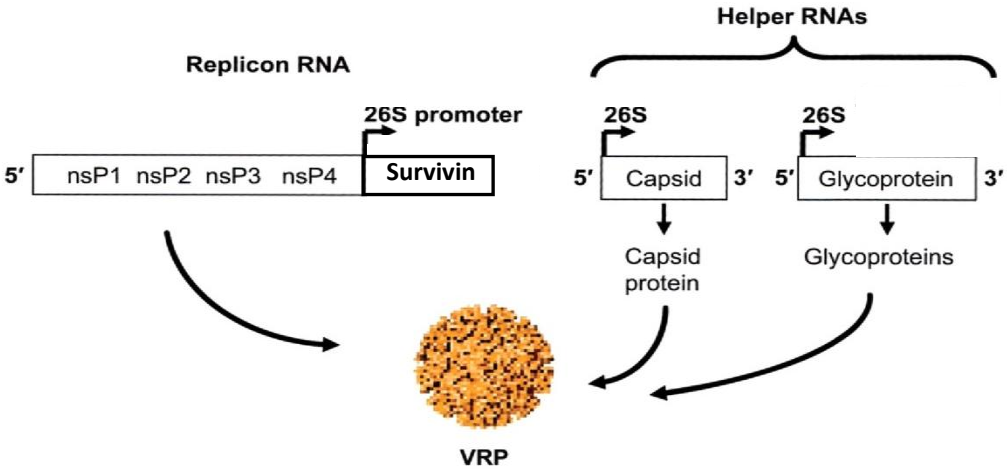

Fig.4 Production of viral replicon particles
For the production of viral replicon particles (VRPs) expressing antigen, packaging cell lines were cotransfected with replicon RNA encoding the antigen (Survivin) and the viral non-structural proteins (nsP1- nsP4) and helper RNAs expressing capsid and glycoprotein genes (Schleiss 2005).

1.1.3.2.3 Non-viral delivery

Non-viral delivery has been explored extensively in the last few years as strategy to avoid viral vectors limitations and to efficiently deliver RNA vaccine inside the cells. Different approaches exist: administration of RNA in a naked form (simply formulated in buffer), or in combination with lipids, polymers, or other compounds. Other methods consist in physical techniques as gene gun or electroporation. Vaccinations based on RNA "naked delivery" show antigen expression and immune response activation, but this method suffers from limited potency, because of RNA instability *in vivo* related to the presence of RNA degradation enzymes in tissues and during storage. Electroporation efficiently delivers the antigen but it presents safety problems. Conversely, synthetic delivery vehicles such as liposome and cationic polymers increase RNA vaccine potency, and do not induce immunity against themselves. Their production is carried out *in vitro*, without the use of cell lines avoiding safety concerns and reducing production cost. Lipid nanoparticle (LNP) formulations represent a good strategy for RNA delivery, because RNA integrity is protected and RNA is functionally delivered in muscle cells. Moreover, LNP that present low surface charge are sequestrated by antigen presenting cells, inducing an increased immune response against the antigen. Other lipid nanoparticles exist and they are under study to improve vaccine efficacy (Geall et al., 2012) (Fig.5).

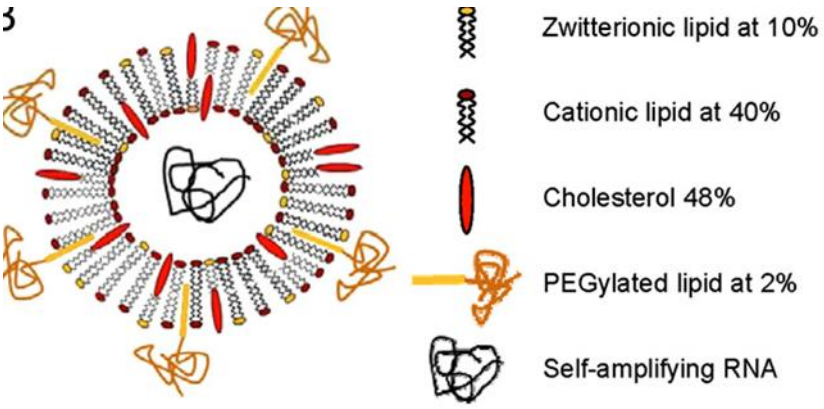

Fig.5 Lipid nanoparticles
Schematic illustration of a lipid nanoparticle (LNP) encapsulating self-amplifying RNA. The percent molar ratios of lipid components are indicated (Geall et al., 2012)

1.2 Survivin: an attractive cancer therapeutic target

Survivin has been selected among a variety of antigens as a suitable TAA, because it is over-expressed by the majority of human cancers, it has a long-lasting documentation in the literature and it has been already tested in different anti-tumor strategies. Its features make it an ideal target antigen for immunotherapy studies by broad spectrum vaccines.

As a 16.5 kDa protein, Survivin is the smallest mammalian member of the Inhibitor of Apoptosis (IAP) protein family. Survivin is encoded by BIRC5 gene located on chromosome 17q25 in humans and on chromosome 11E2 in mice. As demonstrated by X-ray crystallography of the human (Chantalat et al., 2000; Verdecia et al., 2000) and mouse (Muchmore et al., 2000) proteins, Survivin is a stable homodimer in solution and it is structured to form a very unusual bow tie–shaped dimer (Chantalat et al., 2000). It can also function as a monomer both for subcellular localization and for some protein–protein interactions.

Wild-type survivin exhibits a three-intron–four-exon structure in the human and mouse genomes, and it is a 142-amino acids protein. In addition to the wild-type form, two Survivin isoforms are generated by alternative splicing, via insertion of an alternative exon 2: Survivin-2B, or removal of exon 3: Survivin-ΔEx-3 (Altieri 2003). These variants differ in functions and cellular localization (Rodel et al., 2012).

Survivin is a multifunctional protein involved in regulation of apoptosis, in mitosis control and in cellular stress response (Altieri, 2003b). Based on its functions, Survivin localizes in cytoplasm, mitochondria and nucleus. Nuclear Survivin regulates mitosis, in fact it belongs, together with other proteins as Aurora-B kinase, to the Chromosome Passenger Complex (CPC), where it plays a key role in correct chromosome alignment, spindle assembly and correct cytokinesis. Down-regulation of Survivin causes pleiotropic cell division defects, demonstrating that Survivin has a critical role in preserving the mitotic apparatus and allowing normal mitotic progression (Uren et al., 2000).

The mitochondrial pool of Survivin is involved in its anti-apoptotic function. Survivin, in response to cellular stress, is rapidly discharged from mithocondria and released into the cytosol, where it prevents caspase cascade activation, therefore conferring cytoprotection. Recent evidences have demonstrated that Survivin, by inhibiting apoptosis also in endothelial cells, is able to promote neo-angiogenesis, essential characteristic for tumor growth (Xiang et al., 2005; Altieri 2008a). All

these features suggest that over-expression of Survivin helps tumor maintenance and progression (Khan et al., 2011) (Fig.6). Survivin is indeed involved in tumor chemo-resistance and its over-expression is associated with high proliferation, metastasis and poor diagnosis.

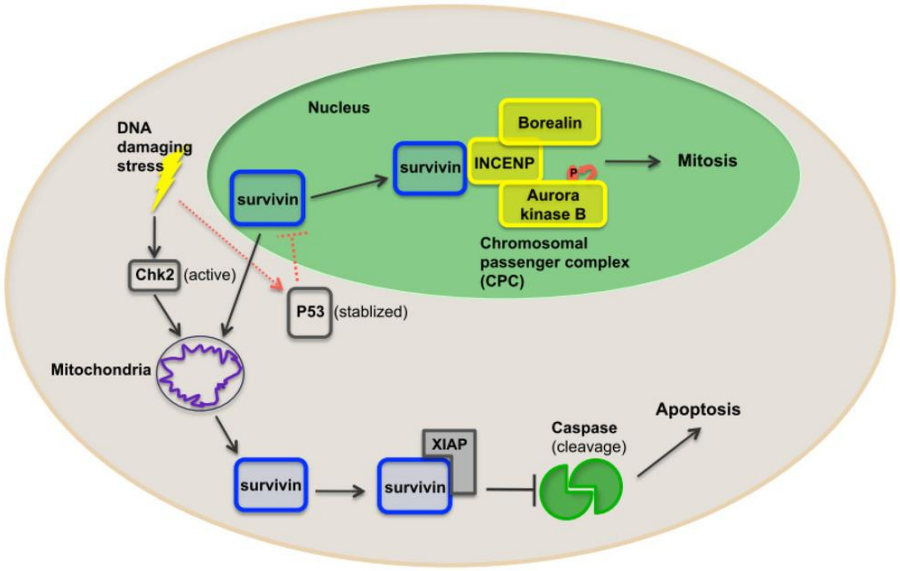

Fig.6 Pathways through which Survivin can favor tumor cell development.
Survivin is a component of the chromosome passenger complex (CPC) and a key regulator of chromosome segregation and cytokinesis. In addition, cell stress signals induce a rapid release of Survivin from mitochondria, inhibiting cell death and promoting tumor cell survival (Mobahat et al., 2014).

Survivin is highly expressed in the embryo and plays an important role in fetal development, in fact lack of Survivin it has been demonstrated to induce embryo lethality (Uren et al., 2000). Conversely, Survivin becomes undetectable in most adult normal tissues (Adida et al., 1998), although recent reports suggest high expression of Survivin in certain rapidly dividing cells (Fukuda et al., 2006) including T-cells (Xing et al., 2004), hematopoietic progenitor cells (Fukuda et al., 2001), vascular endothelial cells (Mesri et al., 2001), liver cells (Deguchi et al., 2002), gastrointestinal tract mucosa (Chiou et al., 2003), erythroid cells (Gurbuxani et al., 2005), and polymorphonuclear cells (Altznauer et al., 2004; Mobahat 2014).

In normal cells, Survivin is highly cell cycle-regulated, through cell cycle dependent and cell cycle independent pathways. Many Survivin inhibitors are tumor suppressors, such as p53, suggesting that repression of Survivin is an important barrier against malignant transformation. An important post-translational modification that controls protein stability and Survivin function is phosphorylation on Thr34 by p34cdc2/cyclin-dependent kinase 1. Survivin Thr34Ala mutant exhibits an increase in protein turnover, due to the loss of stability, and an increase in caspase-

dependent apoptosis. Such Survivin mutant is considered a potential candidate for therapeutic vaccination because it can be more immunogenic than the wild-type form and in tumor pre-clinical studies showed anti-tumor efficacy and increased survival in mice (Mesri et al., 2001b, Yan H et al., 2006, Yu et al., 2010).

In contrast to normal adult cells, over-expression of Survivin has been reported in the majority of human malignancies (Yamamoto et al., 2008) including malignant mesothelioma, pancreatic, ovary, breast cancers and haematological malignancies (Granziero et al., 2001; Nakagawa et al., 2004). The over-expression of Survivin, in the tumor cells cytoplasm may lead to an increased expression of Survivin-derived epitopes in association with MHC class I. These epitopes would be more abundantly presented by MHC-I on tumor cells compared to healthy cells and therefore they could be the targets for anti-tumor cytotoxic T lymphocytes (CTLs) (Bertino et al., 2012). It has indeed been reported in fact that oncologic patients with different types of cancer sometimes show specific-CD8+ T cell responses against Survivin peptides and anti-Survivin antibodies, suggesting that Survivin tolerance could be overcome (or inexistent).

Because of its features, Survivin has been studied in different anti-tumor strategies as cancer therapeutic target (fig.7): antisense oligonucleotides, ribozymes, or siRNA molecules (Pennati et al., 2007), small molecule inhibitors of Survivin function (Xiao et al., 2015), dominant-negative mutants (Mesri et al., 2001), and immunotherapy (Ishizaky et al., 2010; Rapoport et al.,2011). Some of these treatments generated Survivin-specific cytotoxic T lymphocytes (CTLs) presenting biologic effects against tumors and survival increase (Miyazaki et al., 2011; Honma et al., 2009; Bertino et al., 2013). The efforts to develop Survivin-targeted cancer therapies have also reached the clinic with encouraging results (Ryan et al., 2009)

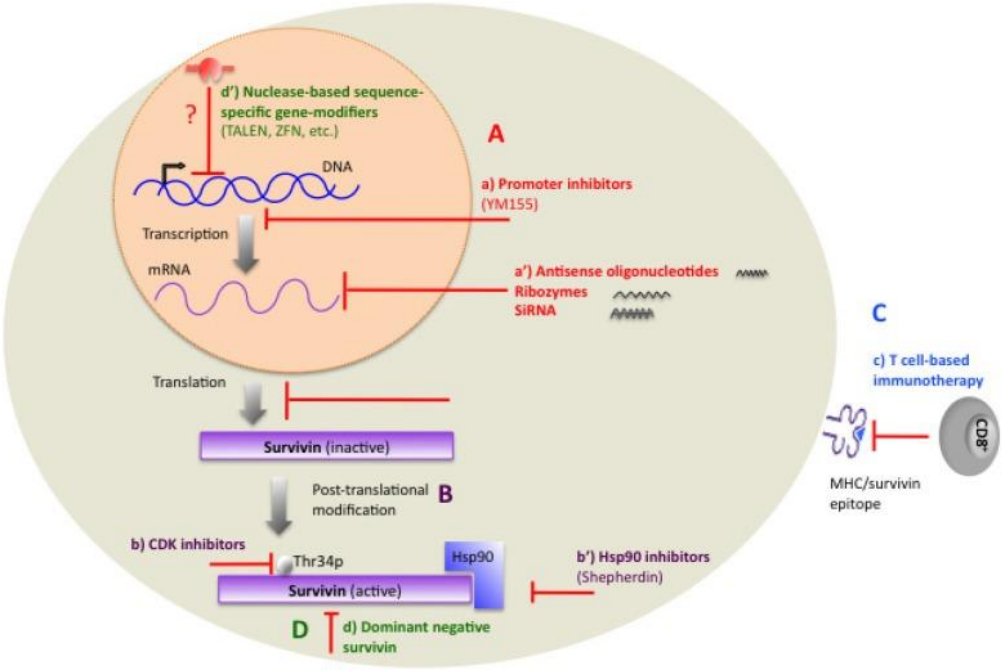

Fig.7 Therapeutic targeting of Survivin.
Survivin-based treatments include drugs that (**A**) function at the transcription level and inhibit the transcription of Survivin, such as promoter inhibitors (**a**), antisense oligonucleotides, ribozymes, and SiRNA (**a'**), (**B**) inhibit Survivin at post-translational level (such as CDK inhibitors (**b**) and Hsp90 inhibitors (**b'**)), (**C**) include vaccines that are based on cytotoxic activities of $CD8^+$ T lymphocytes against specific Survivin epitopes (**c**) or (**D**) gene therapy methods including transfecting with dominant negative mutants (**d**) which encode proteins that suppress Survivin's function. Within this class, it was proposed that use of the breakthrough technology of nuclease-based genome-editing tools (**d'**), may show promising results once designed and applied against Survivin in pre-clinical trials (Mobahat 2014).

1.3 Vaccine-based therapy in mouse tumor models

Mouse models are helpful tools in understanding disease mechanism and in evaluating the efficacy of new treatments. In the present study, the efficacy of VRPs-expressing Survivin was tested in two tumor mouse models: pancreatic cancer and malignant mesothelioma; both are very aggressive, incurable diseases and over-express Survivin. Vaccinations with viral vectors encoding Survivin have already been tested in these mouse models with encouraging results in terms of survival and anti-tumor effects (Bertino et al., 2012).

1.3.1 Malignant Mesothelioma

Malignant mesothelioma (MM) is a rare but very aggressive form of cancer that affects mesothelium, the thin cell wall lining of the body's internal organs and structures. Malignant mesothelioma has three known varieties: pleural, pericardial, and peritoneal mesothelioma. Pleural mesothelioma is the most common type, accounting for about 70% of all MM cases. MM is classified into three forms according to the histological types: epithelial, sarcomatoid, and biphasic (which combines epithelial and sarcomatoid cells). The prognosis of the disease is poor, and the median survival time is 9-12 months from diagnosis. Surgery, chemotherapy and radiation can only extend survival of about 5 months (Harvey ey al., 2005).

Mesothelioma is known to be caused by exposure to asbestos. Asbestos is a natural, yet toxic, mineral that was frequently used in a wide variety of industrial processes and products. Microscopic asbestos fibers enter into the body via the lungs or by ingestion. Once inside the human body, the durable asbestos fibers cannot be broken down or expelled, resulting in a harmful inflammation and scarring of the mesothelium. The latency period between the time of initial exposure and diagnosis is about 30 years. Males are at a much higher risk for MM than females, likely due to occupational exposure **(**Alastair J at al., 2008)**.**

Survivin expression was observed in 91% of human mesothelioma both in nuclei and cytoplasm of tumor cells, probably linked to its pro-mitotic and anti-apoptotic functions (Zaffaroni et al., 2007).

Abstestos causes a similar disease both in human and in mice, therefore the mouse mesothelioma model well recapitulates the tumor characteristics and is a suitable model for treatment tests. For MM induction in mice, different abstestos fibers (crocidolite asbestos, Turkish erionite or US erionite) were injected i.p. in mice resulting in tumor growth. Tumor cells were then isolated from

peritoneal ascites and used for tumor challenge in syngenic mice. Preliminary results have demonstrated that viral vectors encoding for Survivin can confer tumor protection and increase mice survival (Bertino et al., 2012). Despite these encouraging results, viral vectors present a robust neutralizing antibody response against the vector itself after immunization. In the thesis, a peritoneal MM mouse model was employed to test a different technology, VRPs expressing Survivin, aimed at analyzing its efficacy against tumor and overwhelming viral vectors limitations.

1.3.2 Pancreatic cancer

The pancreas, located in the abdomen, is a glandular organ of the digestion system with endocrine and exocrine functions. The exocrine pancreas cells produce enzymes that are released into the small intestine to help food digestion, whereas the neuro-endocrine pancreas cells (such as islet cells) secrete several hormones, including insulin and glucagon, which help to control the sugar levels in the blood. Cancer cells can develop from both types of functional cells, causing different effects. The pancreatic cancer which affects the exocrine part is the most common form. The main causes of pancreatic cancer are smoking, obesity, diabetes and hereditary causes. Recently, pancreatic cancer has been classified as an occupational cancer, because it has been discovered that 1,2-dichloroethane, used in PVC production and extensively used as a degreasing agent and a solvent for paints, can cause the disease (Benson & Teta, 1993).

Pancreatic cancer is the fourth leading cause of cancer death in developed countries, affecting both men and women. The prognosis is poor with a 5-year survival rate for 26% of patients if the cancer is local at the time of diagnosis and less than 5 % if the cancer has metastatized. Despite the development of surgical resection, radiotherapy and chemotherapy, this cancer is highly lethal because it grows and spreads rapidly, and no early diagnosis procedures are available. Moreover, pancreatic cancer is highly chemotherapy and radiation therapy resistant and tumor microenviroinment in pancreatic cancer is highly suppressive (Foley et al., 2015). For these reasons, biomarkers of early detection and novel therapeutic strategies are needed.

Survivin is expressed in 81,25 % of pancreatic cancers (Dong et al., 2015) and malignant tumors express Survivin more frequently than benign ones. It has been reported that pancreatic cancer patients, with positive expression of Survivin in specimens after surgery, had a shorter survival time than those who did not express Survivin, therefore this TAA may become a useful prognostic marker for pancreatic cancer (Kami et al., 2004). Some results also suggest that increased Survivin

and decreased p53 may promote the progression from benign to malignant lesions (Jinfeng et al., 2002).

Genetically engineered and tumor xenograft mouse models have been developed for the study of pancreatic cancer (Din Y et al., 2010). In this study, an orthotopic xenograft mouse model is used, consisting in mouse cancer cells injection in mouse pancreas. This model allows to establish a tumor microenvironment similar to the original tumor and closely reproduces natural human tumorigenesis (Wangiong et al., 2013).

Recent studies showed that also in this mouse model, immunization with viral vectors encoding for Survivin can prolong mouse survival and that adoptive transfer of lymphocytes can confer tumor protection (Piemonti's personal communication; experiments in progress)

2. RATIONALE AND OBJECTIVE OF THE THESIS

Cancer cells are able to express aberrant or elevated level of proteins called tumor associated antigens (TAAs), which can represent the targets of cancer immunotherapy. Cancer vaccines aim at harnessing and enhancing the immune system to specifically target the TAAs expressed on cancer cells, in order to eradicate the tumor. Among tumor antigens, Survivin represents an attractive target for testing different cancer vaccines, because it is over-expressed in virtually every human cancer, and presents multiple functions involved in tumor maintenance and progression. Moreover, Survivin is involved in tumor chemo-resistance and its over-expression is associated with high proliferation, metastasis and poor diagnosis. Oncologic patients with different types of cancers sometimes show CD8+ T cell responses against Survivin peptides and anti-Survivin antibodies, suggesting that Survivin tolerance could be overtaken (or does not exist). In mouse tumor models, viral vector based-vaccines encoding Survivin have been shown to induce tumor protection and increased mouse survival. Nevertheless, viral vectors present different disadvantages and safety problems. The aim of this thesis is to investigate alternative strategies to overcome viral vectors limitations. In particular, we focus our attention on self-amplifying RNA vectors, which are based on an alphavirus genome carrying non-structural genes that encode the RNA replication machinery while the structural genes are replaced with the gene of interest, in this case Survivin. This strategy allows an elevated antigen expression favoring its presentation by MHC class I and II, and leading to an efficient immune response. Self-amplifying RNA expressing Survivin are packaged as viral replicon particles (VRPs) for RNA delivery in mice. VRPs represent a good strategy for the set-up of mice immunization schedules because they are able to infect muscle cells without formulations, they are poorly immunogenic, and are not counteracted by pre-existing immunity against themselves, thus being suitable for repetitive immunizations. In this study, we want to evaluate the efficacy of VRPs expressing Survivin in two different tumor mouse models in term of survival and tumor protection.

3. MATERIAL AND METHODS

3.1 RNA vectors construction and characterization

3.1.1 Cell lines

Baby hamster kidney (BHK)-V cells are fibroblasts cell lines, cultured in Dulbecco's minimum essential medium (DMEM) (GIBCO, Invitrogen Corporation, Milan, Italy) supplemented with 5% fetal calf serum (FCS) (Euroclone Ltd., Pavia, Italy). BHK cells were used for *in vitro* potency assay (IVP), VRPs production and titration, and for characterization of antigen expression through Western blot.

Mouse pancreatic cancer (mPanc02) cells are a non-metastatic tumor cell lines. mPanc02 cells were grown in RPMI 1640 (GIBCO) supplemented with 10% FBS (Euroclone). mPanc02 cells were employed in the challenge of the pancreatic cancer mouse model.

AB1 Luc/c cells are malignant mesothelioma sarcomatoid cells derived from mouse lung, expressing a Luciferase-lentiviral vector. AB1 cells were cultured in RPMI 1640 (with 2mM L-Glutamine + 25mM HEPES) (GIBCO), supplemented with 5% FCS (Euroclone). AB1 cells were used in the challenge of the malignant mesothelioma mouse model.

C2C12 are a mouse myoblast line established from normal adult C3H mouse leg muscle, obtained by ATCC (Rockville, MD). C2C12 cells differentiates rapidly, produces extensive contracting myotubes expressing characteristic muscle proteins, and provides a model for *in vitro* myogenesis and cell differentiation studies. C2C12 cells were used for the immunofluorescence experiments.

All cell lins were maintained in a humidified incubator at 37°C with 5% CO_2.

3.1.2 Mouse Survivin sequence

Wild-type murine Survivin sequence (GenBank: AF115517.1):

atgggagctccggcgctgccccagatctggcagctgtacctcaagaactaccgcatcgccaccttcaagaactggcccttcctggaggactgcgcctgcaccccagagcgaatggcggaggctggcttcatccactgccctaccgagaacgagcctgatttggcccagtgtttttctgctttaaggaattggaaggctgggaacccgatgacaacccgatagaggagcatagaaagcactcccctggctgcgccttcctcactgtcaagaagcagatggaagaactaaccgtcagtgaattcttgaaactggacagacagagagccaagaacaaaattgcaaaggagaccaacaacaagcaaaaagagtttgaagagactgcaaagactacccgtcagtcaattgagcagctggctgccatggatgacctaggttccattccaaacccttgctgggattggactga

Wild-type murine Survivin optimized sequence (purchased from Genescript):

atgggagcaccagctctgccccagatctggcagctgtacctgaagaactacagaatcgccaccttcaagaactggcccttcctggaagattgcgcctgcacccccgagagaatggccgaggccggcttcatccactgccccaccgagaacgagcccgacctggcccagtgcttcttctgcttcaaagagctggaaggctgggagcccgacgacaaccccatcgaggaacacagaaagcacagccccggctgcgccttcctgaccgtgaagaaacagatggaagaactgaccgtgtccgagttcctgaagctggacagacagagagccaagaacaagatcgccaaagagacaaacaacaagcagaaagagttcgaggaaaccgccaagaccaccagacagagcatcgagcagctggccgccatggacgacctgggcagcatccctaacccccctgctgggcctggactga

Both sequence contain the sequence coding for the V5-tag (MDDLGS*IPNPLLGLD*) at C-terminal (Southern et al., 1991).

Codon optimization is a technique based on nucleotidic sequence modification to achieve the highest possible levels of productivity of the antigen production, without altering the aminoacidic sequence. The OptimumGene™ algorithm, used in codon optimization, takes into consideration a variety of critical factors involved in protein expression stages, such as codon adaptability, mRNA structure, and various *cis*-elements in transcription and translation.

3.1.3 Cloning of Survivin in the replicon vector

The sequences of two different mouse Survivin forms (mouse Survivin wild-type (WT) and mouse Survivin WT codon optimized (OPT), each fused at the C-term with the V5-tag) were amplified from intermediate plasmids by PCR reaction using specific primers containing appropriate restriction sites. The primers used were: forward primer 5'–attatGTCGACGCCGCCatgggagctccggcg-3', which contains the SalI restriction site and reverse primer, 5'–attatGCGGCCGCCGCCtcagtccaatcccagcaaa – 3', carrying the NotI site. PCR was carried out with 1 µg of each construct, 10 µM of each primer and KAPA HiFi DNA Polymerase (Kapa Biosystems Ltd.**,** London, UK).

The replicon vector and the PCR products were digested with SalI (5U/µg) and NotI (5U/µg), for 2h at 37°C. Calf Intestinal alkaline Phosphatase (CIP) was added to the vector digestion, for 30 min at 37°C, to remove 5' terminal phosphates, in order to prevent re-ligation of linearized plasmid DNA.

Both digestions were run on an agarose gel to isolate DNA bands, which were purificated through the Wizard® SV Gel and PCR Clean-Up System kit (Promega). Finally, the replicon vector and the different Survivin inserts were ligated. The molar ratio of vector: insert DNA used for ligation was 1:5, using 1 µl of T4 DNA ligase (NEB), 2 µl of 10x T4 DNA ligase buffer in a 20 µl reaction. The reaction was incubated at 16°C over/night (O/N). One µL of each ligation reaction was used to transform using TOP 10 competent cells (Invitrogen). Cells were incubated with DNA on ice for 30 minutes and thereafter heat shocked in the 42°C water bath for 30 seconds. Cells were placed on ice and 250 µl pre-warmed SOC medium was added to each vial. Cells were shaked at 37°C for 1 hour at 225 rpm in a shaking incubator. A volume of 20–200 µl from each transformation was spread on LB agar plates containing kanamycin and incubated at 37°C overnight. The selected colonies were analyzed by plasmid isolation, PCR, and sequencing.

3.1.4 RNA *in vitro* transcription (IVT) and capping

Purified DNA plasmids encoding Survivin forms were linearized, immediately downstream the 3' end of the replicon, by BspQI (2 U/µg) endonuclease digestion for 30 min at 50°C and 20 min at 80°C to inactivate the enzyme. Complete plasmid linearization is an essential step for it employment as template for *in vitro* RNA transcription: circular plasmid templates would generate extremely long, and heterogeneous RNA transcripts since RNA polymerases are very processive. An aliquot of linearized template DNA was analyzed on an agarose gel to confirm complete cleavage. For DNA purification, samples were treated with Phenol/Chloroform/Isoamyl alcohol and centrifuged for 5 min at 13.200 rpm, at 4°C. With this method, proteins are denatured and collected in the organic phase or at the interphase, while nucleic acids remain in the aqueous phase. The aqueous phase was collected and the DNA was precipitated with 1/20th volume of 0,5 M EDTA, 1/10th volume of 3M Na acetate and 2 volumes of ethanol. The samples were chilled at -20° C for at least 15 min, then DNA was pelleted, washed with 70 % ethanol and resuspended in RNAse free water.

One µg of linearized DNA vectors was transcribed using the T7 MEGA Script kit (Life technologies, Grand Island, NY) and incubating the reaction two hours at 37°C. To remove the template DNA 1 µl of TURBO DNase (Life technologies) was added and incubated 15 min at 37°C. Transcribed RNAs were purified by precipitation with of 2.8 M LiCl, and then capped using the ScriptCap m^7 G Capping System (CellScript, Madison, WI) and precipitated again with LiCl. RNA was resuspended

in RNAse free-water and its integrity was evaluated on a 1 % denaturing agarose gel (Ambion-Life Technologies, Grand Island, NY).

3.1.5 Characterization of RNA vectors

3.1.5.1 RNA In vitro potency assay (IVP) and Western blot

The RNA *in vitro* potency (IVP) assay evaluates the capability to RNA to replicate inside the cells and to express the antigen. It is a semi-quantitative test because it compares a new RNA batch with a previously tested standard RNA (a replicon expressing nucleoprotein (NP) of influenza virus). This assay consists in electroporating BHK cells with the newly transcribed RNA, the standard RNA, as positive control or thymus RNA only, as negative control, and each sample is run in triplicate.

The day before IVP, $5x10^6$ BHK cells were plated in T150 flasks and incubated at 37°C for about 28 hours. The day after, for each electroporation, $1x10^6$ BHK cells were washed and resuspended in 250 µl Opti-MEM (Gibco) media. Cells were then electroporated at 120 V, one pulse of 25 ms in a 2mm cuvette (VWR, Radnor, USA) with 4,2 µg of total RNA: 200 ng of each RNA (new batch or the standard) and 4 µg of mouse thymus RNA (Clontech) to reach 4,2 µg of total RNA. Cells were allowed to rest at RT for 10 min, then plated in 6-well plate and incubated over/night at 37°C, 5% CO_2.The day after, cells were resuspended in 400 µl PBS and 100 µl (2,5 x 10^5 cells) were collected to perform western blot (see below) and other 100 µl (2,5 x 10^5 cells) were seeded in duplicate in a 96-round bottom well plate, washed and stained with the viability marker Live/Dead Aqua (Invitrogen) diluted 1:1000 for 20 min at RT in the dark. Cells were washed, then fixed and permeabilized with Cytoperm/Cytofix (BD Bioscience, San Jose, CA) for 20 min at 4°C, washed with Perm-wash buffer (BD Bioscience) and stained with APC-labelled (Zenon® Allophycocyanin Mouse IgG2a Labeling Kit, ThermoFisher) anti-dsRNA antibody (English and Scientific Consulting), as dsRNA is the intermediate product of the RNA vector replication, or with anti-V5 tag antibody (Sigma) at 1:2000 dilution, to test antigen production and to distinguish Survivin expressed by the RNA from the endogenous protein. Stained cells are then acquired by BD FACS Canto II flow cytometer (BD Bioscience) and data are analyzed by the FlowJo software (Treestar, Ashland, OR).

In parallel, Western blot was performed: 2,5 x 10^5 transfected cells were lysed in 1X Lamli sample buffer (62.5 mM Tris HCl pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol). Samples were

boiled at 95°C for 5 min and separated under reducing conditions on a 4-12% Bis-Tris polyacrylamide gel in MES electrophoresis buffer (Life Technologies). Following transfer to nitrocellulose membrane (Life Technologies), Survivin protein was detected using an anti-V5 tag antibody (Sigma) diluted 1:500 in PBS, 3% slim milk 0,1% Tween 20, followed by a HRP-conjugated goat anti-mouse antibody diluted 1:10000 (Dako, Denmark). The bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Milan, Italy) autoradiography.

3.1.5.2 Immunofluorescence

C2C12 muscle cells were plated on coverslips in a 24 multi-well plate ($2x10^5$ cells/well). When subconfluence was reached they were infected with $1x10^6$ I.U. VRPs-mouse Survivin wt or VRPs-GFP as control. Infection was conducted in 1% FBS medium for 4h, thereafter to block VRPs infection, medium was changed and cells were fixed, (with 4% paraformaldehyde solution for 10 min at RT), at different times points to evaluate the time-course of Survivin expression in cells. To permeabilize cells, the samples ware treated with 0,1% Triton solution for 10 min and then cells were incubated with 10% normal goat serum (Life technologies) to block any unspecific binding sties for the secondary antibodies. Primary antibodies: anti V5-tag (Sigma), anti dsRNA (English and Scientific Consulting) and biotinylated Wheat Germ Agglutinin (WGA), each diluted 1:1000 in 0,01% Triton and 3% BSA, were used to stain the cells for 1h at RT. After rinsing, the samples were incubated with fluorochrome-conjugated secondary antibodies diluted 1:2000: Alexa Fluor 647 goat anti-mouse IgG H&L (Abcam) for V5-tag and dsRNA detection, and Streptavidin, or Alexa Fluor® 568 for WGA. Finally, the samples were rinsed and mounted using mounting medium containing DAPI (Fluorescent mounting medium, DakoCytomation). The samples were analyzed at the confocal microscope and stored frozen.

3.2 Viral Replicon Particles (VRPs) production

Viral replicon particles (VRPs) were generated by triple electroporation of BHK cells with the chimeric replicon encoding the antigen and two defective helper RNAs encoding the Sinbdis virus capsid and glycoprotein genes.

Twenty-four hours before cells electroporation, $1x10^7$ BHK cells were seeded in 225 cm^2 cell culture flasks (Corning). The next day, after trypsinization, cells were washed twice with ice-cold PBS, then $6x10^7$ BHK cells were suspended in 500 µl of ice-cold Opti-MEM for each electroporation.

Fifteen µg of each RNA (RNA carrying the Survivin gene and the two helpers RNAs) were added to the cells which were then transferred to a 4 mm gap cuvette (BTX) where they were electroporated twice at 220 V, 1000 µF, with infinite resistance (Electroporator: BioRad GenePulser Xcell). Cells from each cuvette were gently removed and each cuvette was split in two 225 cm^2 flasks. Twenty-four hours after transfection, media containing VRPs were collected and cell debris were spun out by centrifugation for 5 min at 1500 rpm. Supernatants were layered on a sucrose gradient (50% to 20% weight/volume) in 25 x 89 mm UltraClear ultracentrifuge tubes (Beckman) and VRPs were purified by ultracentrifugation (Sw32Ti rotor) at 28000 rpm, for 2 hours at 4°C. Sucrose fraction containing VRPs were then collected. VRPs were washed with Minimal Essential Medium (MEM, Lonza) buffered with 20 mM HEPES and concentrated through Amicon Ultra-15 (Millipore). Purified VRPs were stored at - 80 °C.

VRPs' titration: Viral particles' titration is important to determine the concentration of infectious particles in a stock sample (IU/ml). To titer the VRPs, $5x10^5$ BHK cells/well were seeded into 12 multi-well plates. For a full titration curve analysis of the samples, three five-fold serial dilutions of purified VRPs (1:50; 1:250; 1:1250) were considered. VRPs' dilutions were prepared in 300 µl of DMEM with 1% FBS (to allow the infection) and incubated on cells at 37°C for 15-20 min. Thereafter, 1 ml of DMEM with 1% FBS was added to each well. After 18 h of infection, cells were detached, suspended in 200 µl per well and transferred in 96 multi-well U-bottom plates. Cells were stained as reported in the IVP protocol (see above) and acquired on a BD FACS Canto II Flow Cytometer (BD Bioscience. Data analysis was performed with FlowJo software (Treestar, Ashland, OR). The titer of the VRPs was determined using the following formula: infectious units (IU)/ml = [% of infected cells / (volume of cells / dilution factor).

3.3 Recombinant mouse Survivin protein production

3.3.1 Survivin cloning in pET15b-TEV expression vector

The mouse Survivin sequence was cloned in the pET15b-TEV expression vector by the Polymerase Incomplete Primer Extension (PIPE) method (Klock and Lesley, 2009). PIPE cloning is a ligase-free cloning technology based on the discovery that during the later cycles of normal PCR, as some nucleotides get used up, a population of DNA molecules that are partially single stranded at the 5'-end are generated. By designing complementary 5'-ends that can anneal, and PCR amplifying both vector and insert with these primers, PCR fragments can combine producing recombinant molecules (see Fig. 8).

The primers used in insert-PCR and specific for mouse Survivin sequence were: forward primer, 5' –**CTGTACTTCCAGGGC**atgggagctccggcg–3' and reverse, 5' – **AATTAAGTCGCGTTA**ggcagccagctgctca at –3'.Primers used for vector-PCR were: petTEVfor 5'-**TAACGCGACTTAATT**CTAG CATAACCCCTT GGGGCCTCAAACGG-3' and petTEVrev 5'-**GCCCTGGAAGTACAG**GTTTTCGTGATGATGATGATGATGG CTGCTGCCCATGGTATATC- 3'.

Mixing PCR products (1 µl of each), intermolecular annealing occurs. After transformation in HK100 *E. coli* competent strain, nicks and gaps were then repaired and insert and vector were ligated producing a replicating plasmid. Plasmid DNA was extracted from bacterial colonies by QIAamp DNA Mini Kit (QIAGEN) and the Survivin sequence was checked by sequencing.

The insertion in the PCR pET15b-TEV vector introduced a 6X His-tag at the N-terminus of Survivin.

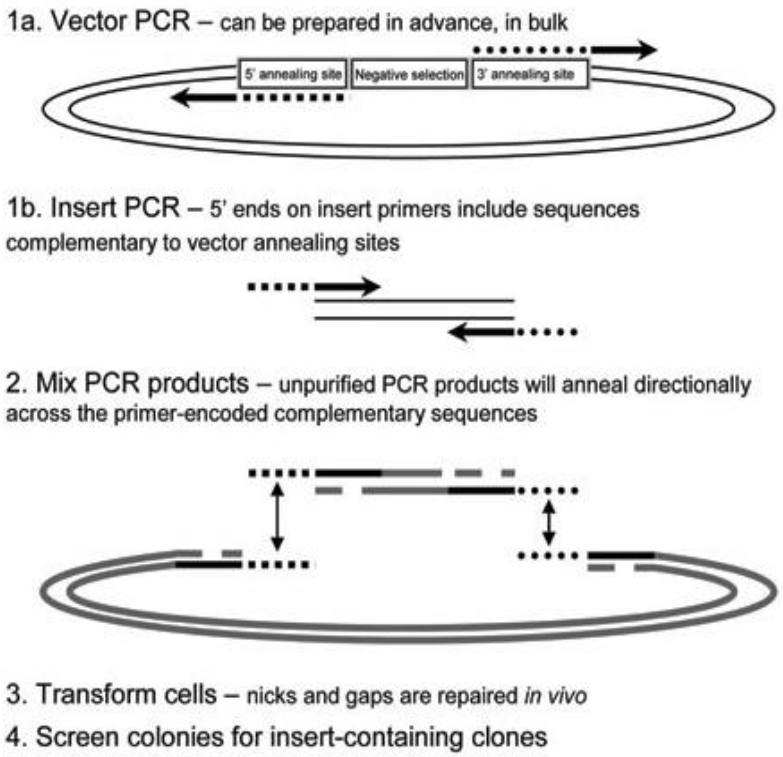

Fig.8 Schematic representation of PIPE cloning method
The thin, black lines indicate the template DNA. The thick, black lines with dashed or dotted ends represent the primers with 5' complementary extensions. The black square dashes represent sequences complementary to each other as do the black dots. The full dark gray lines represent complete strand synthesis and the dashed dark gray lines indicate primer extension resulting from PIPE.

3.3.2 Survivin protein expression and purification

Recombinant Survivin protein was produced by transforming BL21-DE3 cells (NEB) with the pET15b-TEV construct carrying Survivin. Cells were grown in 500 ml LB medium and when the O.D. reached 0,5, isopropyl-beta-D-thiogalactopyranoside (IPTG) (0,5 mM) was added to the culture to induce protein expression. IPTG is a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the lac operon. IPTG binds to the lac repressor and releases the tetrameric repressor from the lac operator in an allosteric manner, thereby allowing the transcription of genes in the lac operon. Addition of IPTG in the culture medium leads to the expression of T7 RNA Polymerase that can bind to the T7 promoter present in the vector. Any foreign gene cloned downstream to this promoter is thus transcribed and expressed.

Protein expression was allowed O/N at 37° C at 200 rpm in a shaking incubator. The next day, the culture was pelleted for 30 min at 6000 rpm and 4°C (F12-6 x 500 LEX Fixed Angle Rotor Thermo

scientific) and the pellet was resuspended in 20 ml Binding buffer (Tris HCL 20 mM, NaCl 300 mM, imidazole 10 mM pH 8). Cells were lysed by ultrasonication, using a sonicator (Qsonica), equipped with a standard probe. The solution was sonicated on ice using 10 cycles of 30 sec on with a break of 2 min interval between the cycles. Cell debris was pelleted for 20 min at 9000 rpm and the supernatant was collected.

As already mentioned, the pET15b-TEV vector adds a hexa-histidine tag to the N-terminus of the protein, exploited in protein purifications. In this study, immobilized metal affinity chromatography (IMAC) method was performed using Ni-NTA (Nickel-NTA) FF resin Agarose column (Qiagen). Nitrilotriacetic acid (NTA) is a tetradentate chelating ligand used in a highly cross-linked 6% agarose matrix. NTA binds Ni+ ions by four coordination sites, so the resin exhibits high affinity and selectivity for 6xHis-tagged recombinant fusion proteins. Proteins bound to the resin are eluted by competition with imidazole.

One ml of Ni-NTA FF was added into a PD-10 empty gravity column (GE), the column was washed with water and equilibrated with binding buffer. The sample was passed through the column, the protein bound the resin and flow trough was collected, then the column was washed with 20 mM and 40 mM imidazole solution. Protein elution was performed using 300 mM imidazole solution (Fig.9). Every step of purification was collected and analyzed on a 4-12% Bis-Tris polyacrylamide gel in MES electrophoresis buffer (Life Technologies) and stained with ProBlue Safe Stain (Giotto biotech) or transferred to nitrocellulose membrane (Life Technologies) for Western blot analysis. Survivin protein was detected using an antibody anti-Survivin antibody (Cell Signaling) diluted 1:500, followed by a HRP-conjugated goat anti-mouse antibody diluted 1:10.000 (Dako, Denmark). The bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Milan, Italy) autoradiography.

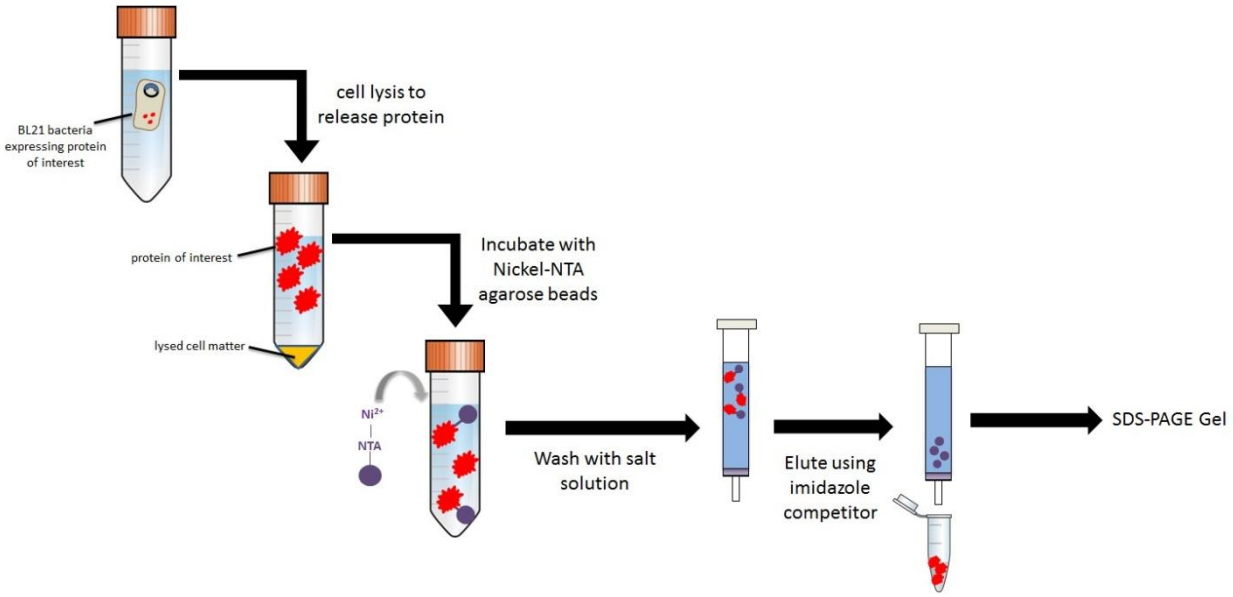

Fig.9 Ni-NTA Agarose column for protein purification
BL21-DE3 bacteria expressing the protein of interest are grown and then lysated to release the protein. The sample is centrifuged and the protein remains in the supernatant that is loaded on the column, washed and eluted. To check the presence and the integrity of the protein, SDS-PAGE gel is performed.

Protein quantification: Bicinchoninic acid (BCA) assay was performed for protein quantification. It relies on the formation of a Cu^{++} protein complex under alkaline conditions, followed by reduction of the Cu^{++} to Cu^{+}. The amount of reduction is proportional to the protein present. Cysteine, tryptophan and tyrosine are able to reduce Cu^{++} to Cu^{+}. BCA forms a purle-blue complex with Cu+ in an alkaline environment, providing a basis to monitor the reduction of alkaline Cu2+ by proteins.

A volume of 980 µl BCA reagent A was mixed with 20 µl BCA reagent B. A volume of 10, 20 or 40 µl of samples or of elution buffer, as blank, was added to the mix solution and incubated at 37°C for 30 min. Color development begins immediately. After 30 min the absorbance at 562 nm was recorded and the protein concentration was determined by comparison to a BSA standard curve.

Buffer exchange: Survivin elution was performed using 300 mM imidazole, but it was not suitable for immunological applications. Buffer exchange from 300 mM Imidazole to PBS was carried out by PD-10 Desalting Columns (GE). Column was washed twice with water, filled up with PBS and the flow-through was discarded. A sample's volume of 1,5 ml was passed through the column by gravity force, and 3,5 ml of PBS was added and the protein was eluted. Typically the recovery is in the range of 70-90%, the BCA assay was used to recalculate sample concentration.

Size exclusion chromatography (SEC): To further purified the recombinant Survivin protein and to eliminate the *E.coli* contaminants, size exclusion chromatography (SEC) was performed. This method allows the separation of the molecules in solution by their size. A volume of 2,5 ml of protein sample was passed into the HiLoad 16/60 Superdex 75 column (GE) with a flow rate of 2,5 ml/min. Samples were collected in different fractions (2 ml/each) by a Fraction Collector Frac-920 (GE). Based on UV absorbance at 280 nm, only expected fractions were analyzed on 4-12% Bis-Tris polyacrylamide gel and stained with Comassie or by Westen blot. . through a column that

LPS REMOVAL: Bacterial endotoxins are lipopolysaccharides (LPS), components of Gram-negative bacteria cell walls known to cause fever and diseases if injected into the bloodstream. Bacterial endotoxins are heat stable and toxicity is not dependent on the presence of the bacterial cells. Since recombinant Survivin protein was produced in bacteria, endotoxin testing was employed to ensure an endotoxin-free product, because for mice treatments LPS must be < 1.0 endotoxin unit (EU)/ml. LPS was measured by Endosafe-PTS test (Charles River) based on limulous amoebocyte lysate (LAL) and if LPS was over the limit concentration in the samples, the EndoTrap Red method (Hyglos) was applied to reduce LPS content.

3.4 *In vivo* studies

3.4.1 Evaluation of the immunogenic potential of VRPs in healthy mice

3.4.1.1 Mice

Five-six week old male C57BL/6 mice, weighing about 20 g, were purchased from Charles River (Hollister, CA). For in vivo experiment in healthy mice, animals were housed in the Novartis Vaccines Animal Facility and experiments were approved and conducted according to the Institutional Animal Care and Use Committee guidelines.

3.4.1.2 Immunization protocol

Groups of 6-week old C57BL/6 mice (6 mice per group) were immunized i.m. 1 or 2-weeks apart with three doses of VRPs encoding for mouse Survivin wild-type (WT) or its codon optimized form (OPT) (10^7 I.U. per dose) diluted in 100 µl PBS. As negative control, mice were treated in parallel with PBS alone. Ten days after the last immunization, mice were euthanized and spleens were collected to perform the immunological assays.

3.4.1.3 Splenocytes isolation

Spleens were crushed using a pestle, pushed through a 70 µm cell strainer (BD Bioscience) and rinsed in washing medium (RMPI, 2% FBS, 1% Penicilin/Streptavidin) by centrifugation at 300 g for 7 min. One ml of red blood cell (RBC) lysis buffer (Biolegend) was added to each pellet and incubated for 2-3 min. Cells were washed as reported before and suspended in complete RPMI (RPMI, 10% FBS, 1% Penicillin/Streptomycin) to be filtered on a 30 µm cup filcon (DB Bioscience), and then counted. Cells were suspended $1x10^7$ cells/ml in complete medium supplemented with beta-mercaptoethanol. Splenocytes of the same immunization group were analyzed individually or pooled together, depending on the assay.

3.4.1.4 Intracellular staining

To measure CD8+ T-cell responses, spleens were harvested and single-cell suspensions were prepared. Splenocytes derived from single mice were plated at 1 $x10^6$ cells/well in 96-well U-bottom plates in RPMI-1640 medium (Gibco-Life Technologies) supplemented with 25 mM HEPES (Gibco-Life Technologies), 10% heat inactivated FBS (low endotoxin; HyClone, Logan, UT), 1X Pen/Strep/Glut (Gibco-Life Technologies) and 50 µM β-mercaptoethanol (Sigma), and stimulated with 5 µg/ml of mouse Survivin derived 15mer-peptides pool (JPT, Berlin, Germany) in the presence of 5 µg/ml of Brefeldin A (Sigma) for 4 hours at 37°C. To measure CD4+ T-cell responses,

splenocytes were treated as described above and stimulated with 15 μg/ml of recombinant mouse Survivin protein. In all cases, plate wells were pre-coated with anti-CD3 antibody and positive control cells were stimulated with anti CD3/CD28 antibodies.

After stimulation, splenocytes were washed twice with PBS and labeled with Live/Dead reagent Yellow (Invitrogen) for 20 min at room temperature (RT) in the dark. Cells were then washed twice with PBS and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) for 20 min on ice. Cells were washed with Perm/Wash solution (Becton Dickinson), therefore blocked with Fc block (anti-CD16/32) (BD Bioscience) for 20 min at RT in the dark. Cells were stained with a mix of the following antibodies: A488-labelled anti-IL4 (eBioscience), A488-labelled anti-IL13 (eBioscience), V450-labelled anti-CD44 (BD), PE-labelled anti-IFNγ (BD), V500-labelled anti-CD4 (BD), PE Texas red-labelled anti-CD8 (BD), PerCP Cy5.5-labelled anti-CD3 (BD), PE Cy5-labelled anti-IL2 (BD) and BV605-labelled anti-TNFα (BD). The cells were incubated with antibodies for 20 min on ice and washed with Perm/Wash solution; finally the cell pellets were resuspended in PBS. Cells were acquired on a LSR II SOS1 flow cytometer (BD Biosciences). To optimize fluorochrome compensation setting, compbeads compensation particles anti-mouse Igk set (BD) were used. Data analysis was done with the FlowJo software (Treestar, Ashland, OR).

3.4.1.5 ELISPOT

Enzyme-Linked ImmunoSpot (ELISPOT) is an assay for cellular immune responses detection: it allows the quantification of cytokines-secreting cells. In this study, IFNγ-specific ELISPOT assay (Mabtech) was performed to measure Survivin-specific T cell response after VRPs-mSurvivin WT immunization. Splenocytes from mice of the same group were pooled and stimulated for 48 h with Survivin-derived peptides pool at the final concentration of 5 μg/ml. Medium was used as negative control and anti-CD3/CD8 as positive control. The cells were seeded in duplicate wells of the ELISPOT assay plates together with stimuli and incubated for 48 h at 37°C, in a 5% CO_2 humidified incubator. Cells were then removed, the wells were washed five times with sterile PBS and stained with biotinylated detection antibody diluted in PBS-0,5 % FBS. The plates were washed as described above and incubated for 1h with Streptavidine-HRP at RT. Wells were washed again, incubated with the TMB substrate solution and developed until distinct spots emerged. Spot development was stopped by rinsing the wells with distilled water. After drying O/N, spots were analyzed by Immunospot Analyzer (Cellular Technology Limited C.T.L., USA)

3.4.1.6 Proliferation assay

The Click‑iT EdU flow cytometry assay was performed. EdU (5-ethynyl-2´-deoxyuridine) is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. Detection is based on a "click" reaction, a copper catalyzed covalent reaction between an azide and an alkyne. In this application, the alkyne is found in the ethynyl moiety of EdU, while the azide is coupled to Alexa Fluor® 488 dye (see Fig.10). Standard flow cytometry methods are used for determining the percentage of S-phase cells in the population.

Splenocytes from mice of the same group were pooled and stimulated in triplicate with the mouse Survivin derived 15mer-peptides pool, the mouse Survivin protein or anti-CD3/CD28 as positive control. For each stimulation, $1x10^6$ splenocyes/well were plated in 96-round bottom plates and incubated with the specific stimulus at 37°C, 5% CO_2, for four days. Thereafter, 10 µM of EdU was added O/N to each sample. EdU gets incorporated into the newly synthesized DNA of proliferating cells in place of thymidine. The day after, cells were washed and stained with Live/dead Aqua (1:1000) for 20 min. After rinsing, cells were treated with FcBlock for 20 min and stained with Pacific Blue-conjugated anti-CD4, and APC-conjugated anti-CD44 APC for 20 min at RT in the dark. Cells were fixed using Cytofix for 15 min and, after washing, were resuspended in PBS-1%BSA. The next day, cells were treated with PBS 1%BSA and saponin for 30 min, in order to permeabilize the membrane, therefore Click-iT reaction reagents (PBS, $CuSO_4$, Fluorescence dye azide Alexa-488 and reaction buffer) were added and incubated for 30 min. After washing, cells were stained with PE Texas red-conjugated anti-CD8 for 20 min and after wash, cells were analyzed with Fortessa. All incubations were performed at RT in the dark. (see Fig.10).

This assay is highly sensitive, it can detect as less as 50-100 proliferating cells and it is non-radioactive.

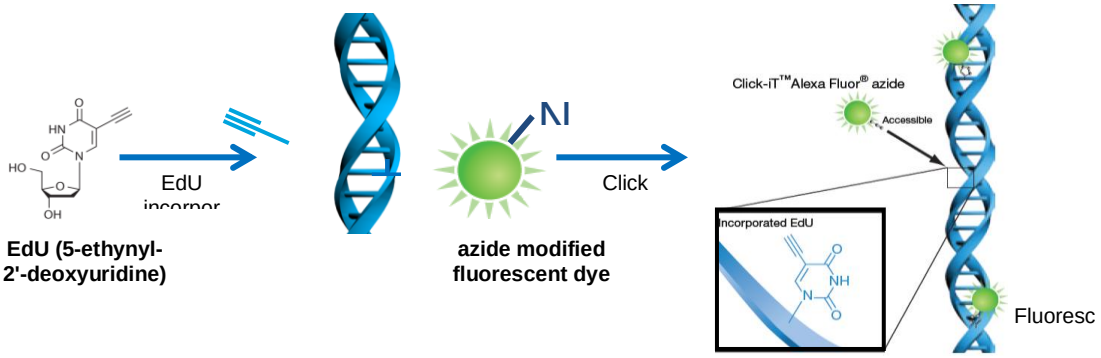

Fig.10 Click-it EdU-based proliferation assay
Edu, a pyrimidine analog, is incorporated in the syntesis DNA. An azide modified fluorescente dye can reacts with EdU and fluorescent signal can be evaluated.

3.4.2 Evaluation of the immunogenic potential of VRPs in mouse tumor models

3.4.2.1 Mice

Five-six week old male C57BL/6 mice, weighing about 20 g, were purchased from Charles River (Hollister, CA). For mouse tumor models, the animals were maintained in the San Raffaele pathogen-free animal facility for one week before experimentation and procedures were performed in accordance with Institutional Animal Care and Use Committee (IACUC n.559- 590)

3.4.2.2 Orthotopic pancreatic cancer model

3.4.2.2.1 Immunization protocol

Immunization with VRPs: 6-week-old C57BL/6 mice were immunized i.m. (in tibialis muscle) 2-weeks apart with 3 doses of VRPs encoding mouse Survivin WT or VRPs encoding GFP as negative control, diluted at $1x10^7$ IU in 100ul PBS/mouse.

Tumor injection: 1 week after the last immunization, mice were challenged with $5x10^4$ mPanc02 tumor cells, injected in the mouse pancreas.

Sub-confluent cultures of mPanc02 cells were treated with 0.25% trypsin, harvested, and washed in pre-cooled PBS. The cells were counted and tested for viability using trypan blue exclusion, and cell viability was always > 95% of total cells. Thereafter, $5×10^4$ cells were resuspended in 50 µl of final solution composed by ice-cold PBS and 25% of Matrigel, for each mouse.

Mice were anesthetized by intra-peritoneal injection of avertin (2,2,2-Tribromoethanol; Sigma-Aldrich) (0.25-0.5 mg/g). After local shaving and disinfection, the abdominal cavity was opened by a 1.5 cm longitudinal incision into the left upper quadrant. The site of tumor injection in the pancreas was identified after lifting the spleen. A volume of 50 µl of cells suspension, or PBS and Matrigel for control mice, were then slowly injected into the pancreatic parenchyma using an ice-cold 27-gauge needle. To prevent cell leakage, the needle was kept in the injection site for 60 sec before removal. Then, the spleen and the pancreas were placed back into the abdominal cavity that was closed by a running two-layer silk suture. Post-operative status and wound healing were monitored every day for one week.

3.4.2.2.2 Mice survival analysis

All animal experiments provided a humane endpoint. Mice were considered for euthanasia (by cervical dislocation) if any of the following conditions occurred: 20% weight loss, tumor interference with the animal's ability to eat or drink, tumor ulceration or infection; if animals became moribund, weak, comatose, unresponsive, showing signs of respiratory difficulty or death appeared imminent.

3.4.2.3 Malignant mesothelioma (MM) model

3.4.2.3.1 Immunization protocol

Immunization with VRPs*:* 6-week-old C57BL/6 mice were immunized i.m. (in tibialis muscle) with 3 doses 2-weeks apart of 100 µl PBS containing $1x10^7$ VRPs encoding mouse Survivin WT or VRPs expressing GFP as control.

Tumour injection: 1 week after the last immunization, $7x10^4$ AB1-B/c-LUC cells (sarcomatoid malignant mesothelioma cells) were injected i.p.

3.4.2.3.2 In vivo imaging system (IVIS)

The *in vivo* imaging system (IVIS) is a non-invasive technique for small animal imaging in pre-clinical settings. Two weeks after cancer cells' injection, mice started to be monitored weekly by IVIS to check tumour growth. Treated mice underwent hairs trimming of the region of interest before being anesthetized by 2% isoflurane-mixed oxygen prior. To generate bioluminescence signals, D-luciferin (potassium salt, PerkinElmer Inc.), a firefly luciferase bioluminescent substrate, was injected i.p. into mice at 150 mg/kg prior to bioluminescence imaging. Images were acquired using the IVIS Spectrum CT (Perkin Elmer Inc.) and were analyzed using the Living Image 4.4 software (Fig.11).

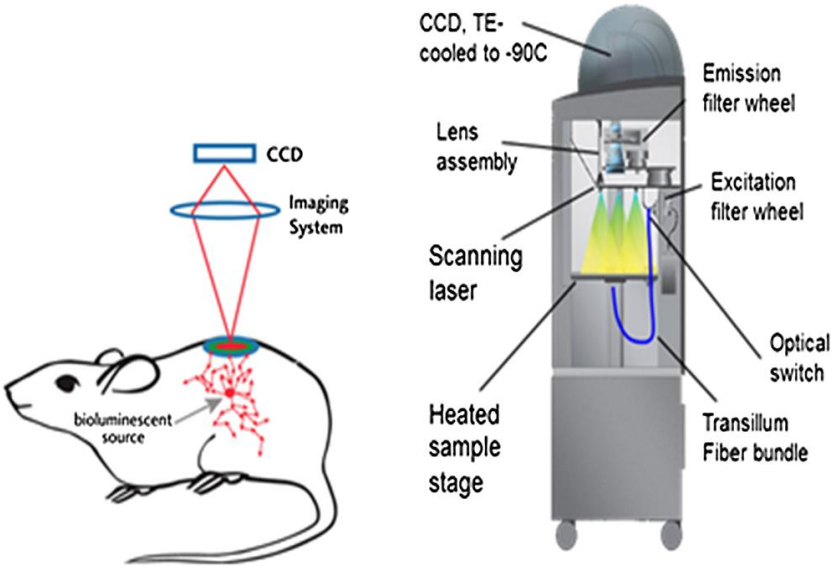

Fig.11 *In vivo* imaging system (IVIS)
IVIS is able to detect the biolumiscence source inside the mice trough a scanning laser and the image is captured by a CCD camera

3.4.2.3.3 Organ removal and tumor dissemination analysis

A necropsy was performed on each animal, and the major organs (peritoneum, mediastinum, liver, spleen, kidneys, stomach, intestine, genital organs, and any detectable lesions including tumors) were collected and evaluated. Tumor masses were measured and counted to determine tumor dissemination in mice treated with VRPs encoding mouse Survivin WT and in control mice.

3.4.2.3.4 Tumor fixation and embedding

Tumor samples were fixed in neutral-buffered formalin. The primary purpose of a fixative is to stop the activity of proteolytic enzymes in the tissue that would digest and damage the sample if not inhibited. The tissues were dehydrated and embedded in a paraffin block. For tissue embedding: samples were soaked into 70% ethanol for 20 min, transferred into 95% ethanol for 20 min and incubation in fresh 95% ethanol was repeated. The samples were then incubated twice with 100% ethanol for 20 min. The tissues were "cleared" by placing them into a xylene bath twice for 20 min to remove the ethanol. The tissues were embedded in molten paraffin wax for 30 min to replace the xylene in the tissues. The mold was filled with paraffin to make a ‘block and then the paraffin was allowed to cool and harden.

Tissue blocks were sectioned by a microtome into slices of 4–5 µm in thickness, cut slices of tissue were placed onto the surface of the water and then picked up on a glass microscope slide. The slides were then laid in an oven at 65 °C for 10–20 min to get rid off the melted paraffin.

3.4.2.3.5 Haematoxylin & eosin (H&E) staining

Hematoxilin and eosin stain is the most widely used stain in histology and it is a useful method to identify cell structures including cytoplasm, nucleus, and organelles and extra-cellular components. The information obtained by this staining allows to diagnose tissues. Hematoxilin binds to basophilic substances, as DNA and RNA, and it is a dark blue or violet stain. Eosin binds to acidophilic substances such as aminoacids and it is a red or pink stain. Sample sections in paraffin were deparaffinized and rehydrated. The sections were stained with hematoxilin for 3 min and rinsed, then stained with eosin for 30 sec and treated with ethanol and xylene. The sections were mounted on a slide and analyzed.

3.4.2.3.6 Terminal dUTP nick end labelling (TUNEL) staining

Terminal dUTP nick end labelling (TUNEL) is a method to detect apoptosis in a tissue section. This method detects DNA fragmentation in the nucleus by terminal deoxynucleotidyl transferase (TdT), an enzyme that incorporate labeled dUTP into 3’ ends generated by the fragmentation of DNA.

To perform TUNEL staining, sections were deparaffinized with 2 incubations in xylene for 5 minutes each, and hydrated with two changes of 100% ethanol for 3 minutes each, and 95% ethanol for 1 minute. After rinsing in distilled water, sections were incubated in 3% H_2O_2 in PBS for 10 minutes to block endogenous peroxidase activity. Samples were pre-incubated in TdT Reaction Buffer for 10 minutes and then in TdT Reaction Mixture for 1-2 hours at 37-40 °C in a humidified chamber. To stop the reaction, sections were rinsed in stop wash buffer for 10 minutes. Samples were incubated with Streptavidin-HRP in PBS for 20 minutes at room temperature. A chromogenic substrate was added for 1-2 min and counterstain with Gill's hematoxylin for 30 seconds. The samples were dehydrated through 95% ethanol for 5min, 100% ethanol for 3min and cleared in xylene for 5 min. The sections were placed on coverslip with xylene based mounting medium.

3.4.2.3.7 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is a method to detect antigens in cells of a tissue section using an antibody against the specific antigen.

For the IHC protocol, sections need to be rehydrated: the slides were immersed in xylene 2 times for 10 minutes each, then placed in 100% ethanol 2 times for 10 minutes each, in 95% ethanol for 5 minutes, 70% ethanol for 5 minutes and finally in 50% ethanol for 5 minutes. The slides were rinsed with deionized H_2O and rehydrated with wash buffer for 10 minutes. To block non-specific staining between the primary antibodies and the tissue, samples were incubated in blocking buffer (1% horse serum in PBS) for 30 minutes at RT. Samples were incubated with antibody anti-Survivin antibody (Cell Signaling) at 1:50 for 30 min. Slides were then washed 3 times for 15 minutes each in wash buffer. The secondary antibody was added for 20 min. Stained sections were rinsed in water, dehydrated, and mounted on coverslips to be analyzed.

3.4.2.3.8 Tumor necrosis score analysis

Tumor necrosis score was calculated according to three parameters: necrosis, vascularization and inflammation. For all tumor sections the P value was calculated by t test between two groups of treated mice (mice immunized with VRPs encoding mouse Survivin WT and control mice) for unpaired data.

4. RESULTS

4.1 Production of RNA vectors carrying different forms of Survivin

4.1.1 RNA vectors' construction

DNA preparation is the first step in the production of RNA. Two different Survivin forms: the wild type (WT) mouse sequence, and its codon optimized form (OPT), were cloned into a replicon vector, which contained the essential elements for *in vitro* transcription (IVT) and for RNA replication (Fig. 12). To this aim, Survivin sequences were amplified from intermediate vectors using primers carrying the restriction sites for SalI and NotI. The recipient vector and the amplified inserts were digested with SalI and NotI, purified and ligated.

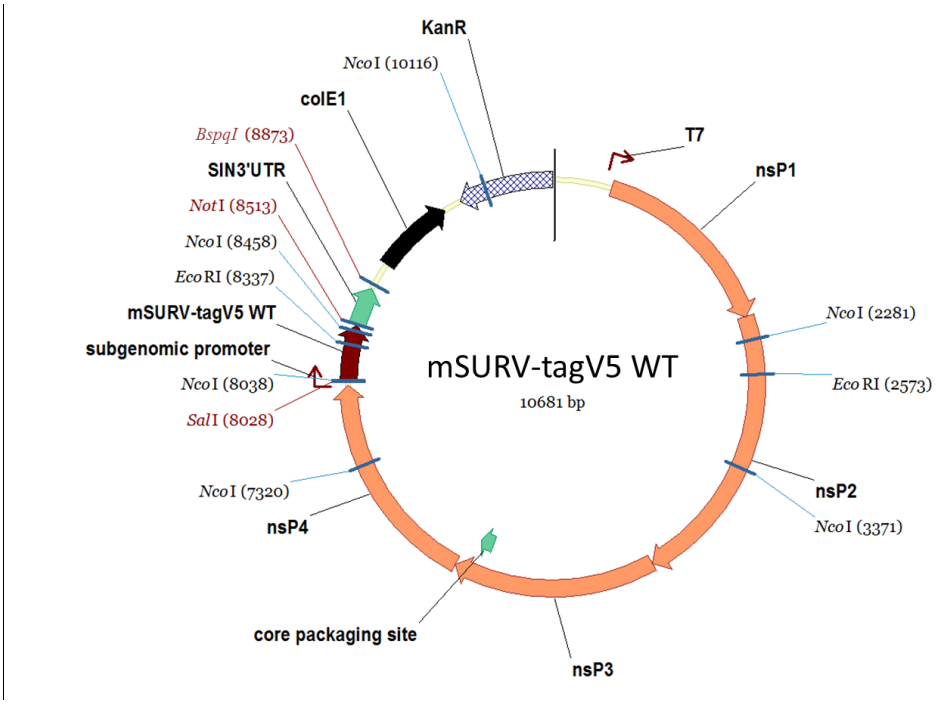

Fig.12 Replicon alphavirus vector encoding Survivin
VCR contains T7 bacteriophage promoter before the four non-structural protein sequences (nsP1, nsP2, nsP3 and nsP4), followed by the Survivin gene (purple arrow). SalI and NotI cloning sites, NcoI and BspQI restriction sites, Kanamycin resistance (KanR) and ColE1 origin of replication are indicated.

The correctness of insert sequences was verified by sequencing and the integrity of the backbone was analyzed by digestion performed with appropriate restriction enzymes, able to cut the vector in crucial regions of the RNA replication portion. Restriction enzyme digestions showed bands of expected weight (Fig. 13), confirming that no chimeric structures were formed during ligation. The wild-type and the optimized Survivin forms were distinguished by the number of EcoRI sites they

contain. As show in figure 13, replicon encoding Survivin WT was cut in two sites by EcoRI and two bands were present, while replicon encoding Survivin OPT contained only one EcoRI site and after digestion only one band was obtained.

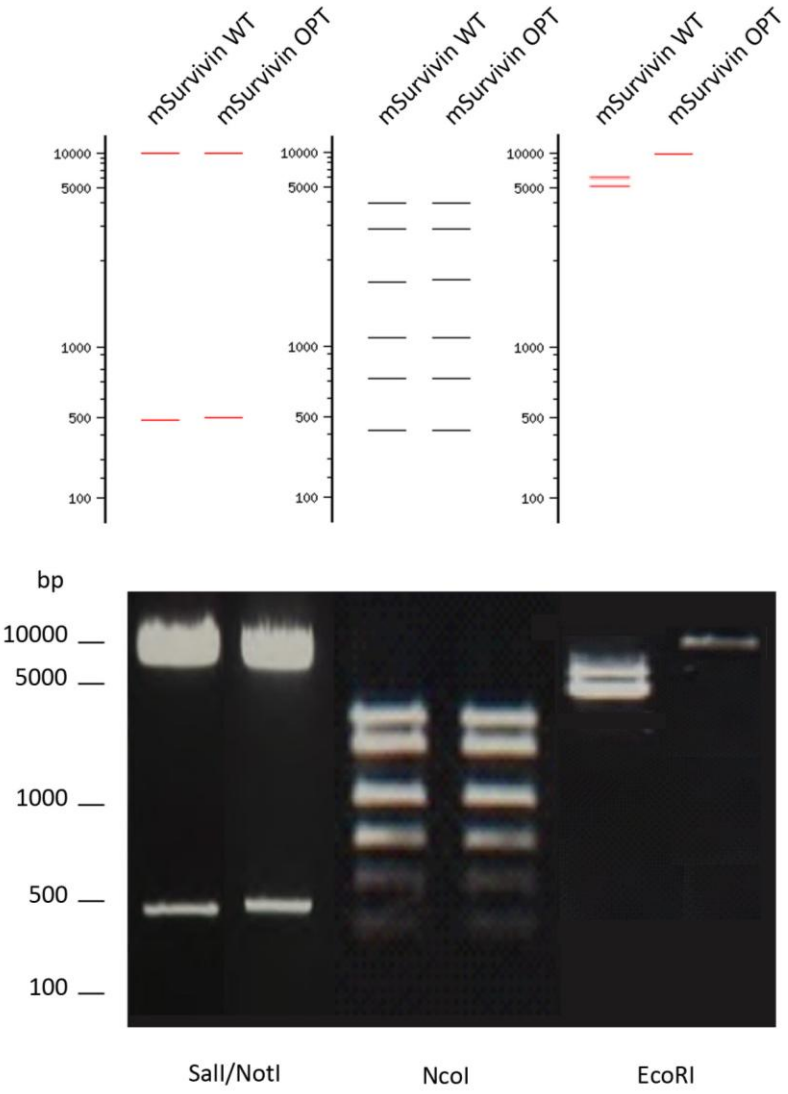

Fig.13 DNA replicons digestion
Replicon encoding wild-type and codon optimized forms of mouse Survivin were cut by NcoI (the first two lanes), SalI/NotI (third and fourth lanes) and EcoRI (last two lanes). Digestions were loaded on 1% agarose gel.

To allow a correct *in vitro* transcription, the replicon vectors carrying the correct sequences were linearized using the BspQI restriction enzyme, which cuts immediately following the 3' end of the

replicon. The complete linearization of DNA sequences was evaluated by agarose gel electrophoresis (see Fig. 14).

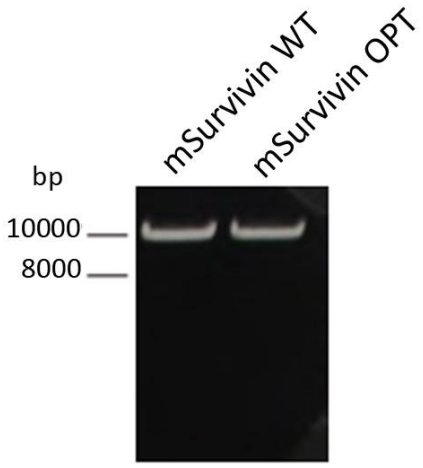

Fig.14 DNA linearization through BspQI digestion
The image shows the replicon vector encoding mouse Survivin WT and its optimized form after linearization with BspQI and loading on agarose gel.

Linearized DNA templates were purified and *in vitro* transcribed into RNA, through a T7 RNA polymerase reaction. The synthetized RNA was then capped, by adding a 7'-methylguanylate (m^7G) at its 5' end, in order to be transcribed correctly.

4.1.2 Characterization of RNA vectors

The purified RNA was characterized by different techniques to check its ability to replicate and to express the antigen, prior to be subjected to further *in vitro* and *in vivo* applications. After transcription and capping reactions, the RNA integrity was verified by agarose gel electrophoresis: as shown in Fig.15a, the RNA bands were of the expected size of about 10000 bp, and showed no signs of degradation. All RNA constructs were tested by *in vitro* potency assay (IVP) to control their ability to replicate inside the cells and to produce the antigen. BHK cells were electroporated in parallel with RNA carrying the different mouse Survivin forms and the transfection efficiency was compared to a standard RNA of known potency. Sixteen hours after transfection, cells were stained with an antibody anti-dsRNA, to check the percentage of cells containing replicating RNA, or with an antibody against V5-tag, to discriminate Survivin protein expressed by RNA replicons from endogenous protein. Stained cells were then analyzed by FACS.
As shown in Fig.15c, the percentage of dsRNA positive cells was comparable between Survivin-RNAs and positive control, represented by cells transfected with standard RNA, confirming that

both RNA constructs encoding Survivin were able to replicate inside the cells. The capability of RNA to produce the antigen was confirmed by V5-tag positive cells (not shown), whose percentage was comparable to that of dsRNA positive cells. To further confirm Survivin expression, a Western blot analysis was performed on total cellular lysates using anti V5-tag antibody (Fig.15c).

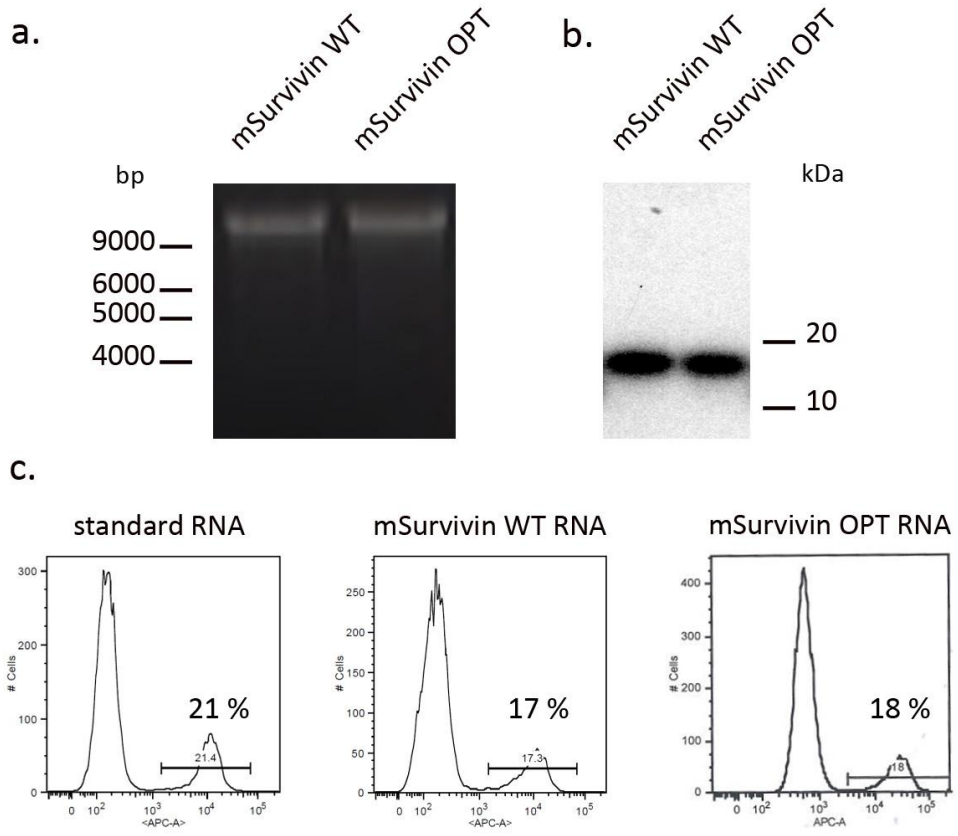

Fig.15 Characterization of RNA vectors encoding mouse Survivin forms
a. The agarose gel electrophoresis displays RNA integrity and RNA bands with the expected weights (about 10000 bp). **b**. The Western blot performed on total lysates of BHK cells transfected with RNA encoding mouse Survivin WT or optimized and staining with anti-V5 antibody (1:500) showed bands of the expected weight (16 kDa) **c**. IVP assay performed on cells transfected with standard RNA (left panel), RNA encoding mouse Survivin WT (mSurvivin WT) (central panel), and RNA encoding optimized Survivin form (mSurvivin OPT) (right panel). The percentage of dsRNA positive cells indicated above the dsRNA positive peak.

4.1.3 Viral replicon particles (VRPs) production

In this study, VRPs were used to deliver RNA *in vivo*. VRPs were obtained by co-electroporation of BHK cells with RNA carrying Survivin genes and two RNA helpers that provide structural proteins *in trans* allowing VRPs packaging. VRPs released in the supernatant were purified by a sucrose gradient and their titer was defined by infecting BHK cells with serial dilutions.

To investigate the kinetics of replication of RNA inside muscle cells and Survivin expression, infection of murine C2C12 muscle cells was performed. Cells were incubated with VRPs carrying mouse Survivin WT. Infection was allowed to proceed for 4h, followed by medium replacement in order to block further VRPs entry. Cells were subsequently fixed at different time points: 4h, 24h and 48h post-infection (p.i.) and then stained with anti-dsRNA and anti-V5 tag antibodies. Confocal microscopy was used to analyze the cell staining.

As shown in Fig.16, dsRNA was detectable inside the cells' cytoplasm already 4h p.i. and was still visible at 48h p.i.. Survivin protein expression peaked at 24h p.i. and was consistent with RNA replication. These results confirmed that VRPs were suitable for RNA delivery in muscle cells.

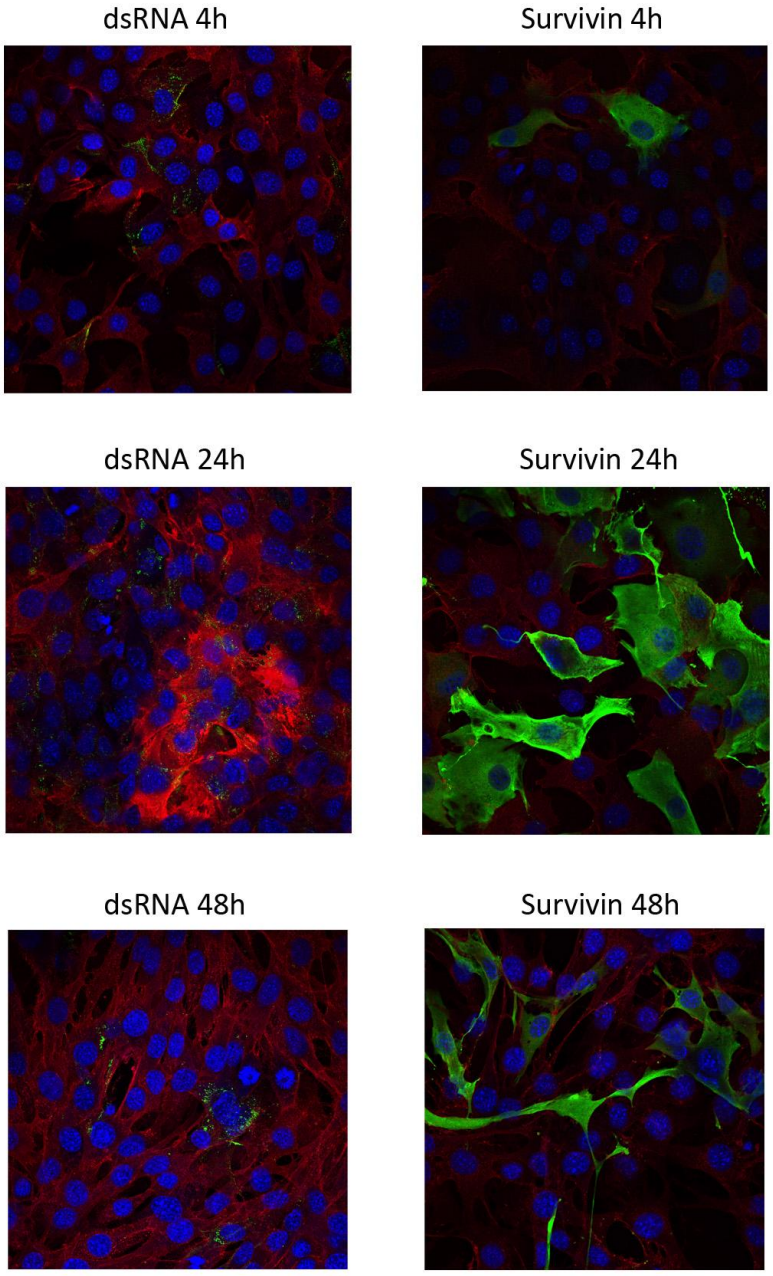

Fig.16 Immunofluorescence of RNA replication and Survivin expression in muscle cells
The panels show C2C12 muscle cells infected with VRPs-mSurvivin WT, at different timepoints(4h, 24h and 48h) and stained with anti-dsRNA (images on the left) and with antibody against the V5-tag that identifies Survivin expressed by VRPs (images on the right). Nuclei are stained blu by DAPI and cell membranes are stained red by lectin antibody. DsRNA is stained green by anti-dsRNA antibody (left images) and Survivin is stained green by anti-V5 tag antibody (righ images)

4.2 PRODUCTION OF RECOMBINANT SURVIVIN PROTEIN

4.2.1 Recombinant Survivin protein expression

Recombinant mouse Survivin protein was produced to be used as splenocytes stimulus in immunological assays. Mouse Survivin wild-type gene was subcloned in the pET15b-TEV expression vector (Fig.17) through PIPE, a ligase-free cloning technology.

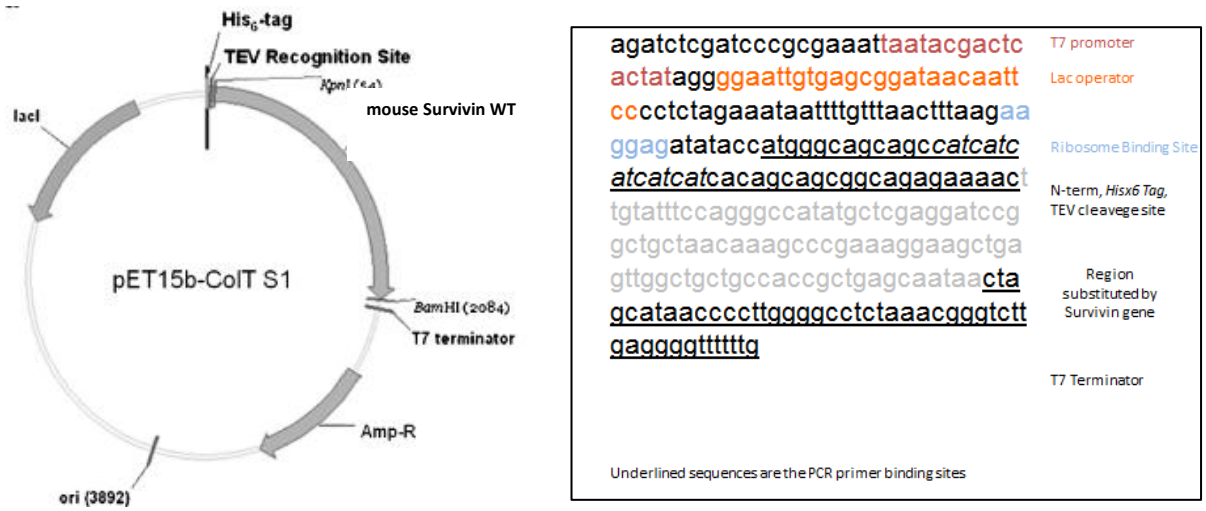

Fig.17 pET15b-TEV expression vectors
The pET15b-TEV vector contains a gene coding for ampicillin resistance (Amp-R), the lacI gene from the lac operon that codes for the lac repressor (LacI), the T7 promoter DNA sequence, the lac operator DNA sequence, and the ribosome binding site.

In the pET15b-TEV vector, transcription of the Survivin gene is blocked by the Lac repressor. Addition of IPTG (isopropyl b-D-1-thiogalactopyranoside) inactivates the Lac repressor and allows transcription by the T7 RNA polymerase (coded by the host bacteria BL21-DE3) from the T7 promoter to the T7 terminator. The construct was transformed into *E. coli* BL21-DE3 cells, where high levels of Survivin protein expression were obtained after addition of the IPTG inducer. To verify protein expression, bacteria lysates, pre- and post-IPTG induction, were collected and loaded on SDS-PAGE gel. As shown in Fig.18, after induction a band of Survivin was present at the expected height.

The eluted protein was quantified and controlled with Comassie gel (Fig. 18a) which displayed a band of the expected size and Western blot analysis confirmed the identity of the purified protein (Fig.18b). Analysis of the protein elution showed that it was not suitable for mice immunization or used in immunological assays because it presented *E.coli* contaminants. Thus, to further purify the

recombinant Survivin protein, size exclusion chromatography (SEC) was performed using the immobilized metal affinity chromatography (IMAC) method taking advantage of hexa-histidine tag added by pET15b-TEV vector to the C-terminal of the protein. This method enables the collection of several fractions of the initial sample and though a graph, indicating absorbance at 280 nm, to evaluate the peaks relative to all the sample components. The fractions corresponding to defined peaks were analyzed by Westen blot and only the fraction containing the Survivin protein (in this case fraction 15) (Fig. 19) was collected and employed for immunological studies.

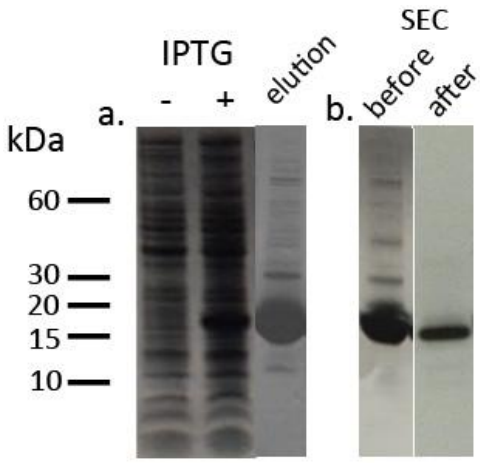

Fig.18 Recombinant Survivin protein expression and purification
The pET15b-TEV encoding mouse Survivin recombinant protein was grown in E. coli BL21-DE3 strain. (a) Protein expression was induced by IPTG and pre- and post-induction lysates were evaluated on Comassie gel (first two lanes). Protein was purified and eluted sample was evaluated by Comassie gel (third lane). (b) Western blot was performed to confirm Survivin protein elution before and after SEC.

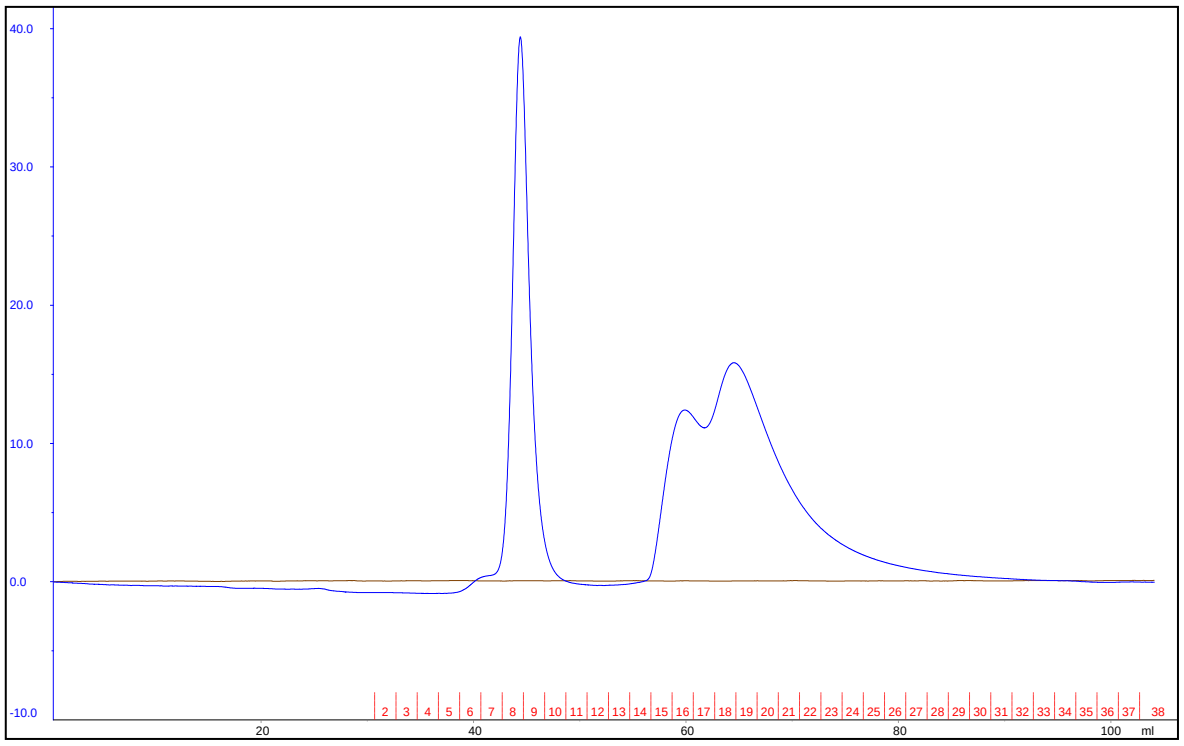

Fig.19 SEC purification
The graph shows the peaks obtained by SEC purification. The blue line represents UV absorbance at 280 nm and indicates protein passage. The brown line indicates conductance. In the lower part of the graph, the number of the collected fractions is reported (red numbers).

Give that protein elution was performed in 300 mM imidazole solution, a buffer change from imidazole to PBS was required before using the purified protein as immunological stimulus or for protein immunization. Therefore, Survivin protein was passed through a PD10 desalting column and eluted in PBS.

Finally, LPS contamination was evaluated by the Limulus Amebocyte Lysate (LAL) test, because LPS can interfere with splenocytes' stimulation, causing aspecific activation of cells. If LPS levels in the sample were > 1.0 Endotoxin Unit (EU) /ml, the protein preparation was subjected to the EndoTrap system for LPS removal.

4.3 IN VIVO EXPERIMENTS

In order to evaluate the capability of VRPs expressing Survivin to confer protection against tumor, two different mouse experimental settings were evaluated. The capability of VRPs encoding Survivin to elicit an immune response was first evaluated in healthy mice. Different schedules were tested to select the best immunization protocol to be used in the tumor challenge setting. As reported in literature, it is difficult to obtain a strong specific immune response against TAA by immunotherapy, because they are self-proteins. This first experiment aimed to evaluate whether the RNA vaccine delivered by VRPs enabled this immune response and to define the best immunization schedule on the basis of the experimental data.

For tumor challenge and protection experiments, two representative tumor models were selected: pancreatic cancer and malignant mesothelioma.

4.3.1 Immunogenic analysis of VRPs expressing Survivin in healthy mice

Based on the literature, two different immunization schedules were tested. Six-week old male C57BL/6 mice were immunized i.m. with three doses of VRPs encoding mSurvivin WT (10^7 IU/mouse), 1 week or 2 weeks apart. Mice splenocytes were collected ten days after the last immunization and the cellular immune response against Survivin was tested. Splenocytes were stimulated *in vitro* by mouse Survivin derived 15mer-peptide pool and purified recombinant Survivin protein, then the immune response was evaluated by IntraCellular Staining (ICS), ELISPOT, and proliferation assays. No differences between vaccinated and control mice were detectable by ICS and ELISPOT (data not shown). Therefore, more sensitive assays to detect even low responses were required.

The proliferation assay was performed by stimulating splenocytes *in vitro* for 4 days with a pool of overlapping 15-mer peptides covering the entire Survivin sequence, or medium as control. A thymidine analog, that is incorporated in newly synthetized DNA of the proliferating cells, was added to the samples. In the same samples, cellular markers (anti-CD4 and anti-CD8 antibodies) allowed identification of the proliferating cell populations. As shown in Fig. 20, an increase in CD4+ T-cell proliferation was evident in splenocytes from mice vaccinated every 2 weeks, while no proliferation was observed in splenocytes from mice vaccinated 1 week apart, indicating that the

best schedule of immunization was 3 doses administered 2 weeks apart. CD8+ T cells did not show any increase in proliferation (not shown).

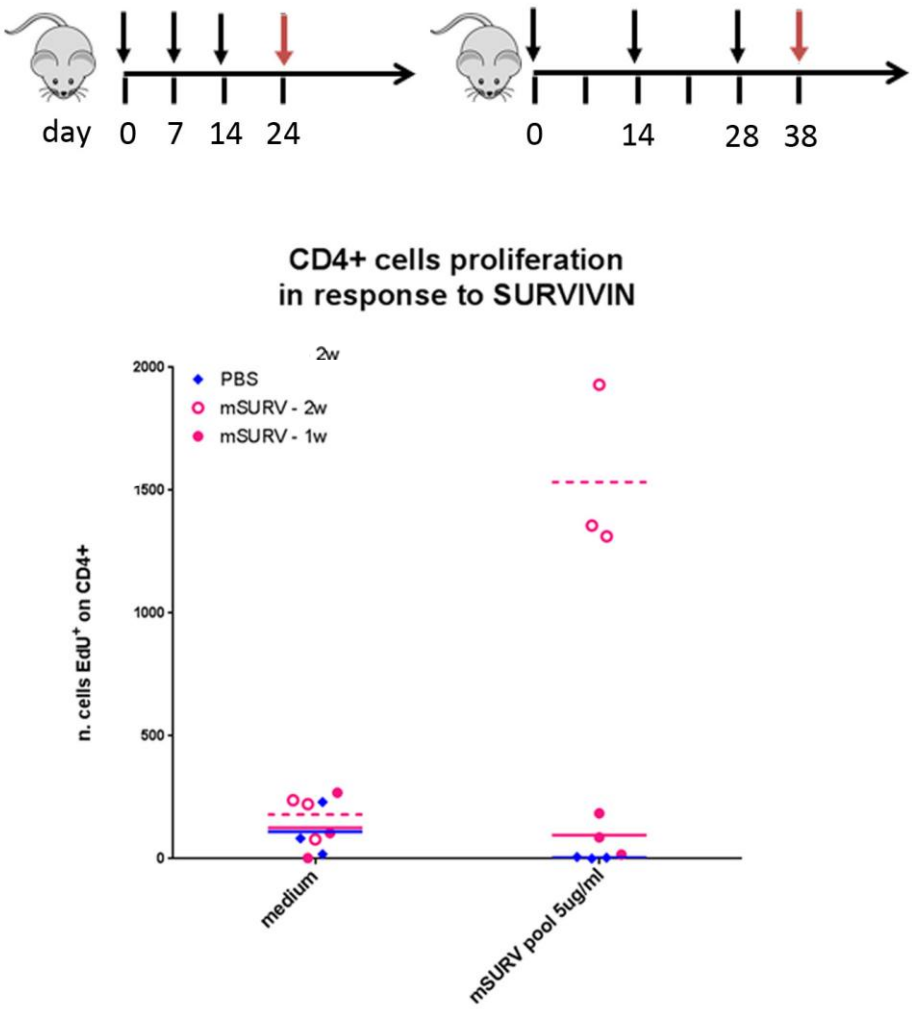

Fig.20 Proliferation assay analysis
The upper panel represents the two immunization schedules: three immunizations (black arrows) administered 1 week (left image) or 2 weeks (right image) apart. Ten days after the last immunization mice were euthanized and their splenocytes were collected (red arrow). In the lower panel, CD4+ T cell proliferation results are depicted. Cells were pooled and stimulated in triplicate with mouse Survivin derived 15mer-peptide pool or medium as control. Bars represent median value of the triplicates. A negative control, PBS was administered every 2 weeks.

In the next protocol, mouse Survivin WT was compared to mouse Survivin codon optimized to assess if sequence optimization could improve the immune response. Mice were immunized i.m. with three doses of VRPs-mSurvivin WT or VRPs-mSurvivin OPT (10^7 IU/mouse) administered 2 weeks apart. ICS and ELISPOT assays were performed on splenocytes as described above and again, in agreement with our previous results, no differences were appreciable between the two groups with these assays. In the proliferation assays, the splenocytes were stimulated with

Survivin recombinant protein which is a more appropriate stimulus for CD4+ T cells. As reported in Fig.21, the results confirm that CD4+ T cells proliferate in response to the stimulus but no differences between Survivin WT and its codon optimized form were observed.

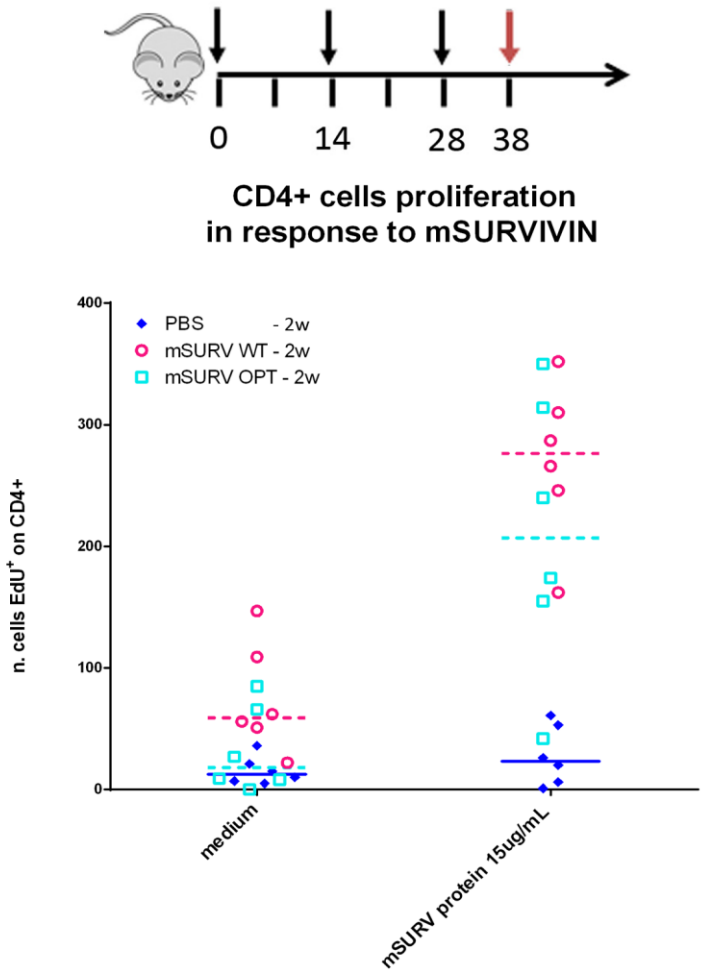

Fig.21 Proliferation assay analysis
The immunization schedule is displayed above the graph and shows three immunizations administered 2 weeks apart. Ten days after the last immunization mice were euthanized and the splenocytes were collected. The lower panel, shows CD4+ T cell proliferation results. Cells were pooled and stimulated in triplicate with mouse Survivin recombinant protein or medium as control. Bars represent median value of the triplicates. A negative control, PBS, was administered every 2 weeks.

Since the codon optimized form was not superior to the WT form, which is commonly used in studies reported in literature, this form was selected for the following tumor models immunization.

4.3.2 *In vivo* experiments in tumor mice models

After the identification of the best immunization schedule, the efficacy of VRPs encoding mouse Survivin WT in tumor prevention was evaluated. The well characterized pancreatic cancer and malignant mesothelioma mouse models were chosen for VRPs testing. These models show features similar to human cancers and noteworthy, other immunotherapeutic strategies expressing Survivin have already been tested in these models with encouraging results. In particular, several studies concerning viral vector based-therapies, such as MVA and FPV expressing mouse Survivin WT, reported an increased overall survival and a reduced tumor progression in treated mice compared with control mice (Bertino et al., 2012, Yu-Qian Wang et al., 2013). Based on these evidences, our experiment aimed to evaluate whether VRPs encoding RNA vaccine could show results comparable to those found in the literature with other vectors. The schedule of immunization used in the study was the one with three doses of VRPs administered two weeks apart and survival and/or tumor progression was evaluated.

4.3.2.1 Pancreatic cancer model

Six-week old male C57BL/6 mice (10 mice/group) were immunized three times with two weeks intervals with 10^7 I.U./mouse of VRPs-mSurvivin WT or VRPs-GFP as control. Seven days after the last immunization mice were challenged with pancreatic cancer cells mPanc02 ($5x10^4$cells/mouse). After tumor challenge, mice survival was evaluated.

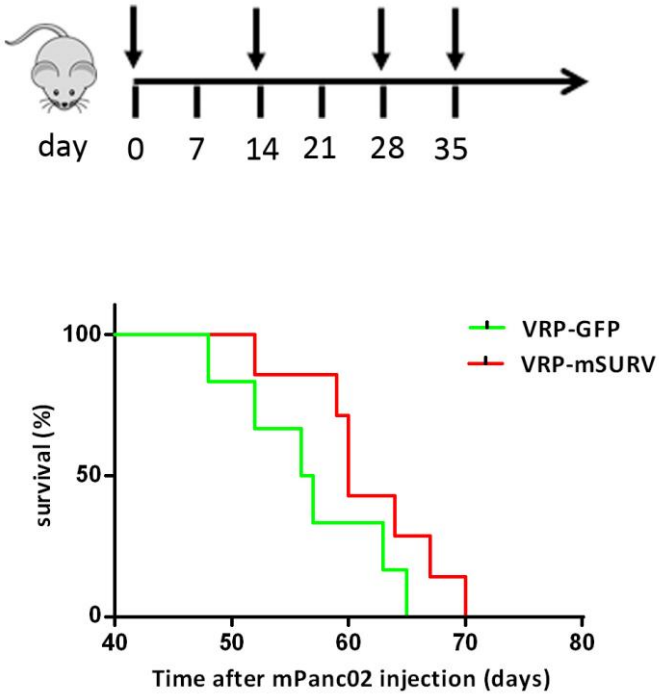

Fig.22 Survival in pancreatic cancer model after VRPs-mSurvivin treatment
Upper panel: immunization schedule in which the mice were injected with three doses of VRPs (10^7 I.U./mice) every 2 weeks and one week after the last immunization the mice were challenge with mPanc02 cells.
Lower panel: the graph represents the survival curves of VRPs-mSurvivin WT treated mice (red line) compared to control mice immunized with VRPs-GFP (green line)

Mice survival curve analysis showed an overall small increase of survival in treated mice when compared to controls. This difference was not statistically significant, possibly due to the small number of mice employed, however the trend showed that VRPs-mSurvivin WT mediated an increased survival (Fig.22).

4.3.2.2 Malignant mesothelioma model

In malignant mesothelioma model, tumor progression was evaluated through *in vivo* imaging analysis, in order to appreciate even small differences in treated and control mice. In addition to mice survival also *ex vivo* analysis of tumor masses was performed.

Six-week old male BALB/c mice were immunized with 10^7 I.U./mice VRPs-mSurvivin WT or VRPs-GFP as control, three times two weeks apart. One week after the last immunization mice were challenged i.p. with AB1 mesothelioma cells.

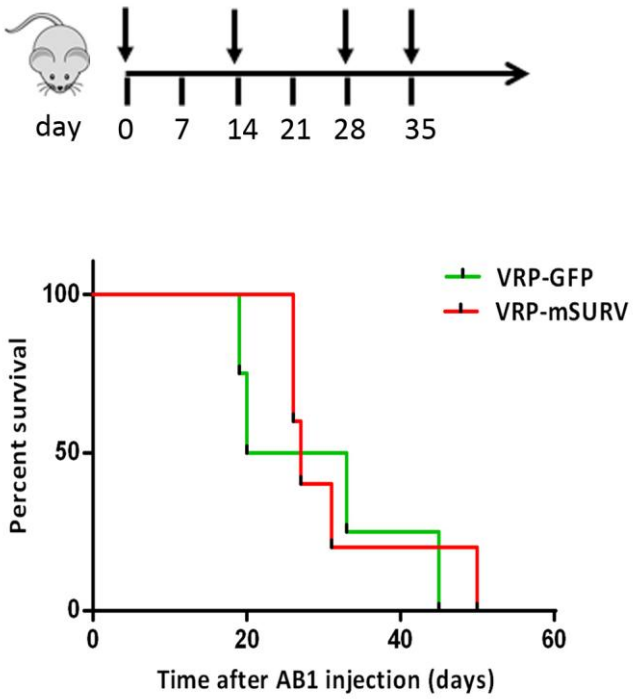

Fig.23 Survival analysis of mice treated with VRPs-mSurvivin WT in malignant mesothelioma model
Mice were immunized with three doses of VRPs mSurvivin WT or VRPs-GFP two weeks apart, then one week after the last immunization the mice were challenged with tumor cells. The graph show mice survival after tumor injection: mice treated with VRPs-mSurvivin WT are identified with the red line and control mice with the green one.

Mice survival analysis showed an initial survival advantage in treated mice. However this trend was present only for a week, then the difference vanished and the two survival curves became undistinguishable (Fig.23). This data suggested that the treatment might have some effect in the early phases of tumor development.

Tumor progression was evaluated also by the *in vivo* imaging system (IVIS) two weeks after challenge. IVIS allows to detect the bioluminescence signals released by AB1 cells carrying luciferase after luciferin injection in mice.

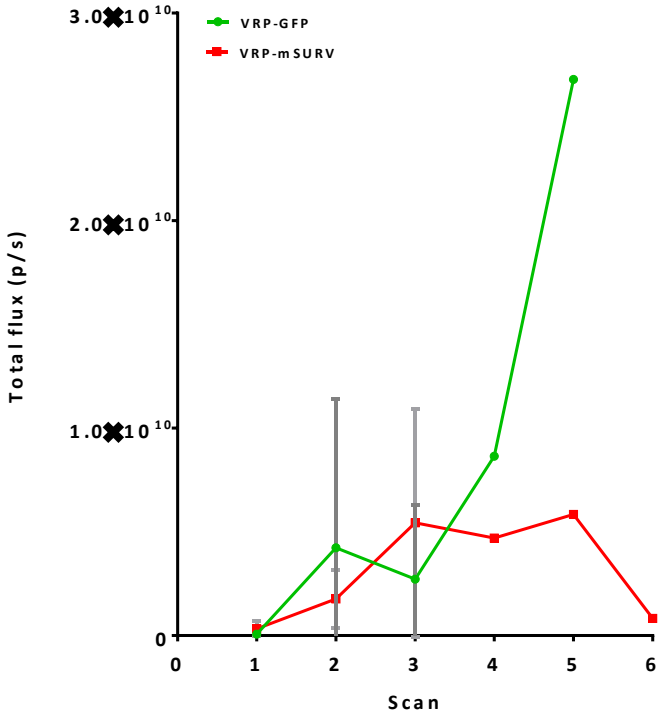

Fig.24 IVIS analysis of tumor progression
The graph shows the average IVIS signals of VRPs-mSurvivin WT treated mice (red curve) and control mice (green curve) during six scans.

IVIS analysis pointed out that VRPs-mSurvivin WT immunized mice presented a reduced tumor progression compared to controls especially in the latest scans (fig.24)

After being sacrificed, mice were analyzed for tumor dissemination, evaluated by counting the amount of independent masses found inside the peritoneum of each mouse (Fig.25). Despite there was no difference in mice survival between treated mice and controls, tumor progression in mice immunized with VRPs-mSurvivin WT was decreased (Fig. 25a). Indeed, the mean number of masses per mouse was reduced of approximately 50% in the treated mice. Moreover, tumor masses were formalin-fixed and paraffin-embedded to allow thin sections of samples allowing histopatologic analysis. After hematoxylin and eosin staining intra-tumor necrosis, vascularization and inflammation were evaluated in each section. In particular, a score from 0 to 3 was attributed for each parameter, and a tumor necrosis score was determined adding together the values (as shown in Fig.25b).

Histologically, the tumors presented themselves as a neoplastic mass circumscribed by a pseudocapsule, where the neoplastic cells had a high degree of differentiation characterized by cellular pleomorphism and a prominent nucleus. Cells undergoing mitosis and a moderate number of giant cells were observed. The tumors of mice vaccinated with VRPs encoding mSurvivin presented more vascularization than control mice. The presence of inflammatory cells seemed to be higher in treated mice and necrotic areas were more abundantly present. Therefore, mice treated with VRPs-mSurvivin WT displayed an slightly increased tumor necrosis score in their tumors, suggesting that vaccination induced an effect of the immune system against the tumors. These results indicated a biological effect of VRPs-mSurvivin WT in tumor dissemination and in intratumoral necrosis.

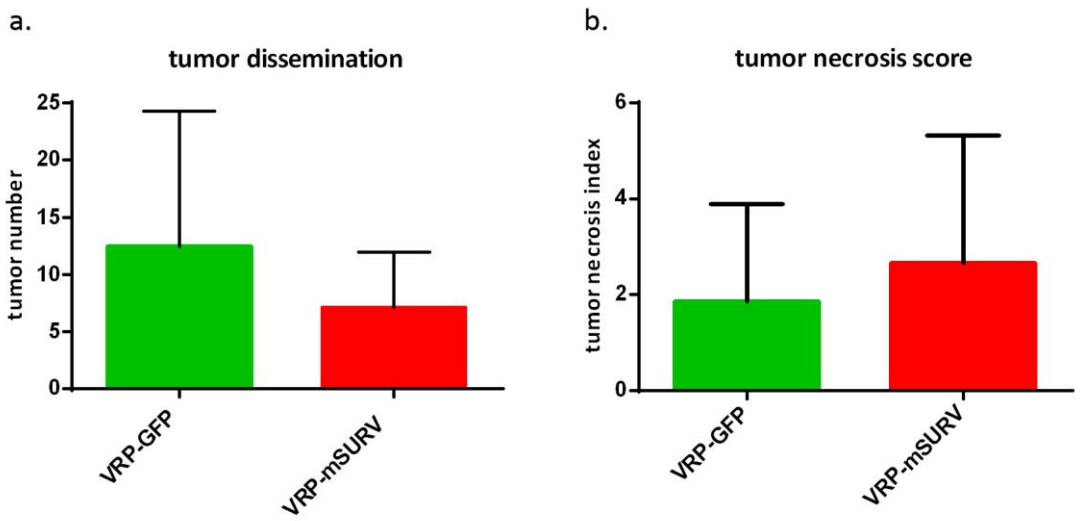

Fig.25 *Ex vivo* analysis of tumor masses
a) The graph shows the average of tumor dissemination in VRPs-mSurvivin WT treated mice (red column) and VRPs-GFP control mice (green column). b)The graph presents the tumor necrosis score of tumor masses from VRPs-mSurvivin immunized mice (red column) compared to control mice (green column).

To further confirm intra-tumoral necrosis, the TUNEL assay was performed on tumor sections. As shown in Fig. 26, intra-tumoral death in treated mice was significantly higher than in control mice. This result confirmed that VRPs-mSurvivin induced an anti-tumor effect.

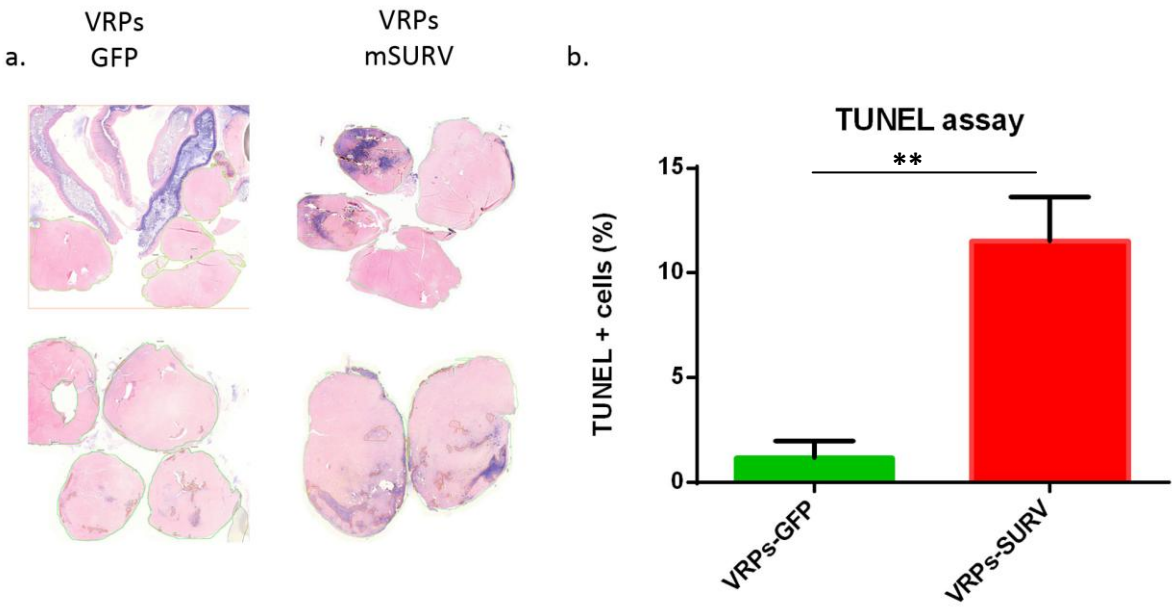

Fig.26 TUNEL analysis in tumoral masses
The panels showe the TUNEL results of VRPs-mSurvivin WT and VRPs-GFP treated mice. (a) TUNEL staining in masses and (b) percentage of TUNEL-positive cells .

5. DISCUSSION

Cancer cells are characterized by high cell proliferation, resistance to cell death stimuli, induction of angiogensis, activation of tissues invasion and metastasis, mechanisms of immune system evasion and genetic instability resulting in over-expression or modification of self-proteins, called tumor associated antigens (TAAs) (Hanahan et al., 2011; Jones and Baylin, 2002). In normal conditions, the immune system has the natural capacity to detect and destroy abnormal antigens to prevent tumor formation, but cancer cells develop various mechanisms to evade immune response (Mapara et al., 2002). Therefore, the main aim of immunotherapy is to activate the immune system against TAAs to overcome tolerance against these self-protein, harnessing and enhancing an immune response to specific targets thus leading to the killing of cancer cells (Aguilar et al., 2011).

Vaccination represents an attractive strategy to elicit specific immune responses against TAAs. The major difficulties in cancer immunotherapy are: 1)to optimize TAAs presentation by dendritic cells, 2) to develop obtain a boost of the weak immune response against TAAs, usally poor immunogens, and 3) to overcome the immunosuppressivion in the tumor bed. To this aim, different vectors for antigen delivery have been studied. Viral vectors represent a sensible vehicle due to their high efficiency in gene delivery and in immune response activation. Viral vector (especially Poxvirus)-based vaccination has been widely used for a long time for preventing infectious diseases, as in the case of the smallpox vaccine that succeeded in the eradication of the disease, and also in experimental setting for AIDS and influenza treatment. It has been widely used also in cancer treatment protocols, showing the ability to induce tumor protection and increase in survival both in pre-clinical models and in clinical trials. However, viral vectors present various limitations such as a possible pre-existing immunity against the vector, and the induction of a neutralizing antibody response against the vector itself after immunization, therefore multiple vaccinations with the same vector become inefficient (Larocca et al., 2011).

To overcome viral vector limitations, different approaches have been considered, such as protein-, peptide-, DNA- or RNA-based vaccines. In this thesis, a self-amplifying RNA vaccine has been studied as a novel approach in cancer treatment.

Self-amplifying RNA is based on an alphavirus genome, which contains four non-structural genes encoding the replication machinery, while the structural genes, required in infectious alphavirus particle production, are replaced with the transgene (immunogen) of interest. Alphavirus genome-based self-amplifying RNA is a single positive strand RNA, which, once entered into the cell, is translated in the cytoplasm producing the RNA replication machinery. This allows the amplification of the genome itself and the expression of the encoded transgene. This system enables high protein production leading to efficient presentation of antigenic peptides by MHC class I and II, inducing high levels of both humoral and cellular immune responses. The self-amplifying RNA strategy presents several advantages compared to viral-vectors: it avoids induction of an immune response against the vector itself, allowing repetitive immunizations. Moreover, the RNA remains confined in the cytosol, avoiding safety concerns on nuclear integration and oncogenic transformation. Furthermore, double-stranded RNA (dsRNA) production during RNA vector replication stimulates the innate immunity by activating Toll-like receptor (TLR) signaling, and helping the adaptive immune response. Several published studies in the literature report that alphavirus replicon based-vaccines have been widely employed as cancer treatment in pre-clinical and clinical trials, eliciting elevate antibody and cellular responses against tumor antigens (Geall et al., 2012).

Among TAAs, Survivin has been chosen as a model antigen, because it is over-expressed in the majority of human cancers, while in normal cells it is almost undetectable. Survivin presents multiple features, because it is involved in both mitotic regulation and in the main anti-apoptotic mechanism (Altieri, 2003b). Over-expression of Survivin in cancer cells, preventing apoptosis, helps tumor maintenance and progression. Therefore, it represents a good target for immunotherapy, especially because tumor cells become "addicted" to high levels of Survivin reducing the probability of escape mutations (Mobahat 2014).

The aim of the project was to study the efficacy of self-amplifying RNA encoding Survivin in tumor protection and survival in mice tumor models.

The first part of the project was focused on the production of replicon constructs encoding for Survivin. Mouse Survivin, in its wild type sequence or in its codon optimized form, was cloned into the replicon vector to be *in vitro* transcribed from DNA to RNA. The ability of the RNA constructs to replicate inside the cells and to express the antigen was demonstrated by *in vitro* potency assay

and Western blot analysis. Moreover, constructs suitable for Survivin protein expression in bacterial hosts were produced. The purified protein was employed for immunological assays.

The self-amplifying RNA vaccine can be delivered inside the cells in different manners: as naked RNA, as viral replicon particles (VRPs) or by nonviral delivery systems. Naked RNA shows efficacy in mouse models, but it has instability problems due to RNA degradation. Nonviral delivery systems, based on liposomes, are able to fuse with cellular membranes and release RNA in the cytoplasm. These are the optimal delivery strategies because they completely avoid the immunogenic response against the vector. Moreover, their production is carried out *in vitro*, without the use of cell lines, thus avoiding safety concerns and reducing production costs.

In this thesis, to first evaluate if the self-amplifying RNA technology could represent an efficient anti-cancer therapy, VRPs have been chosen for delivery, because they represent the gold standard to test new immunological constructs. Iin particular alphavirus derived VRPs do not show an immune response against themselves, allowing repetitive immunizations (Uematsu et al., 2012). VRPs are able to infect several cell types, such as muscle cells and APCs, without the need of further formulations, they are suitable for testing immunization schedules, and for setting-up new experimental approaches. Moreover, VRPs are considered safe, since once they deliver the RNA inside the cells they do not spread in the host (Perri et al., 2003; Maruggi et al., 2013). For VRPs production, replicon constructs carrying mouse Survivin, in its wild-type (WT) sequence and in its codon-optimized (OPT) form (modified to increase its production), were transfected in a packaging cell line, together with two helper RNAs encoding the alphavirus capsid and glycoproteins, to enable particles' production. VRPs were then tested *in vitro* and it was demonstrated that they were able to replicate inside the cells and to express the Survivin protein.

The second part of the thesis aimed at testing the immunogenicity of VRPs mSurvivin WT and mSurvivin OPT in healthy mice, choosing the best immunization schedule and Survivin form. Based on the literature, C57BL/6 male mice were immunized with three doses of VRPs mSurvivin WT administered one or two weeks apart. Survivin-specific immune responses were not detectable through standard immunological analysis. Conversely, a proliferation assay (a more sensitive test), revealed proliferation of Survivin-specific CD4+ T-cells, but not CD8+ T cells, only in splenocytes of mice immunized two weeks apart with VRPs mSurvivin, indicating that this schedule works better in eliciting an immune response.

To evaluate if the sequence optimization could increase antigen-specific immune responses, immunizations with both VRPs-mSurvivin WT and VRPs-mSurvivin OPT were performed in parallel. The proliferation assays showed comparable CD4+ T cells proliferation levels for both constructs. Given that the codon optimized form was not superior to the wild-type form, which is commonly used in the literature, only the latter was selected for being further investigated in tumor models.

In the last part of the project, efficacy of VRPs-mSurvivin WT was tested in two mouse tumor models: pancreatic cancer and malignant mesothelioma. These should be suitable models for studying VRPs ability in tumor protection and mice survival, because they are both well-characterized models, recapitulate disease features comparable to human cancers, and over-express Survivin. Moreover, viral vectors expressing Survivin have been previously tested in both models with encouraging results.

In the pancreatic cancer model, immunization with VRPs-mSurvivin WT induced an initial advantage in survival, with a delay of about 7 days in the onset of mice death. Unfortunately, this trend was lost during tumor progression and the survival curves of vaccinated mice and non-vaccinated controls started to overlap. This weak survival increase suggested that an effect of VRPs-mSurvivin WT could be restricted to the initial phase of tumor proliferation.

In the malignant mesothelioma model, mice were immunized with the same protocol of the pancreatic cancer model and tumor progression, mice survival and *ex vivo* tumor features were analyzed.

As observed in the pancreatic cancer model, mice treated with VRPs-mSurvivin WT present only a slight increase in survival in the first days after tumor injection; then the difference is lost. *In vivo* imaging system (IVIS) analysis showed a decreased tumor progression in VRPs-mSurvivin WT treated mice compared to control mice, which presented an higher bioluminescence signal derived from tumor cells mostly in the latest part of cancer development, suggesting again that Survivin vaccination delayed tumor progression. Upon euthanization, tumor dissemination was evaluated and treated mice showed a reduction of approximately 50% in the mean number of masses when compared to control mice. Moreover, histological intra-tumoral analysis carried out on hematoxilin-eosin stained tumor sections revealed a certain level of tumor necrosis in tumors of treated mice, suggesting that VRPs-mSurvivin treatment can induce death of tumor cells. To further confirm tumor necrosis, cancer sections were stained with TUNEL, which detectes cellular

death. A significantly higher intra-tumoral death was observed in tumors of mice treated with VRPs mSurvivin compared to control tumors. Intra-tumoral cell death by hemorragic necrosis was also documented by P.Bertino, University of Hawaii, in the same mesothelioma tumor model, following MVA-Survivin vaccinations (Fig. 27).

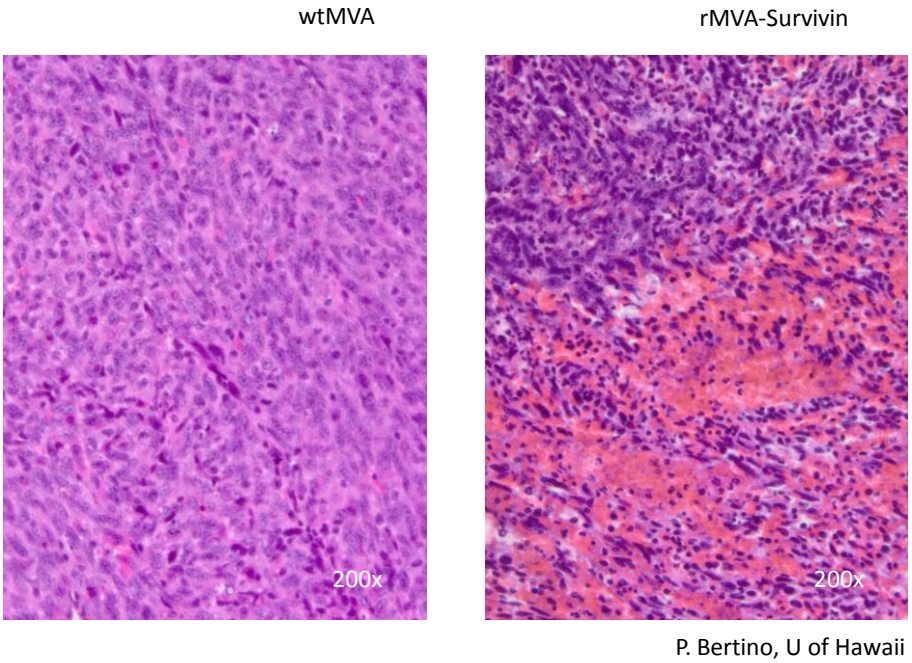

Fig. 27 Intratumoral necrosis in the mesothelioma mice model
The figure shows two tumor sections stained with hematoxil and eosin. The left image refers to tumor of control mice (immunized with empty MVA) , while the right image shows a tumor section of mice treated with MVA encoding Survivin. In the latter section hemorragic necrosis and inflammatory cell infiltration are evident.

These results suggest that VRPs-mSurvivin are capable of conveying a biologic effect against the investigated tumors, although overall survival of treated mice was not increased. A possible explanation of the limited effect of the vaccine could be attributed to mechanisms of tumor escape. This hypothesis was supported by recent results in the same mesothelioma model (by P. Bertino): mice treated with viral vectors encoding Survivin (in a therapeutic setting), showed a limited increase in survival, but they died of the tumor. Immunohistochemistry demonstrated a layer of Survivin-negative tumor cells at the perifery of tumors. This finding explains why the vaccine-induced response exerts only a limited effect on tumor suppression, with no significant differences in long-term mice survival.

In conclusion, the results obtained suggest that VRPs-mSurvivin WT seem to have a biological effect on tumor development in terms of a reduced number of tumor masses and an increased

intra-tumor death, indicating that VRPs-mSurvivin represent a partially efficient strategy that should be combined with other anti-tumor therapies (such as chemotherapy) to reach higher vaccine efficacy.

As reported in the literature different preclinical studies and clinical trials based on cancer vaccine strategy have been performed with different tumor associated antigens and despite the induction of a specific immune response against the tumor, the outcomes have been limitated (Buonaguro et al., 2011; Xo et al, 2003). There are many reasons for these results such 1) the immune tolerance against the tumor associated antigens or 2) the limited cytotoxic T lymphocytes (CTL) expansion due to the activation of regulatory T lymphocytes; 3) moreover the tumor could activate various mechanisms of immune escape or 4) the induction of high-affinity adaptive immunity is inefficient. To overcome such limitations several approach can be adopted: including in the vaccination protocol inflammatory cytokines, as interferon (IFN-α) and interluekin-2 (IL-2), or combine cancer vaccines with therapeutic interventions aimed to eliminate and/or control Treg (Emadi et al., 2009). Moreover, alternative promising strategies involves the combination of checkpoint inhibitors with therapeutic vaccines (Mahoney et al., 2015), this would allow the activation of a patient's pre-existing anti-cancer immune response (Pardoll., 2012).

In particular, cancer immunotherapy targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or programmed cell-death protein 1 (PD-1), and programmed cell-death 1 ligand 1 (PD-L1) represent attractive strategies beacuse they have shown clinical activity in different types of cancer (Sharma P et al., 2015). CTLA-4 is a molecule expressed by activated T cells, competing with CD28 signaling on T cells, that downregulates the immune system. Ipilimumab, a monoclonal antibody against CTLA-4, was used to treat melanoma patients improving overall survival (Hodi et al., 2010).

PD-1 is expressed on activated T and B cells, it plays an important role in down regulating the immune system by preventing the activation of T-cells. PD-1 has two ligands, programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2). PD-L1, its primary ligand, is expressed on a subset of hematopoietic and non-hematopoietic cells and is regulated by pro-inflammatory cytokines. The upregulation of PD-L1 and other factors is correlated with an immunosuppressive microenviroinment. The immunosuppressive tumor microenviroinment is one of the primary reason of failure of most immunotherapies (Mahoney et al., 2015).

Multiple reports of anti-PD-1 therapy have shown promising results in clinical trials, expecially in treating patients with melanoma and other malignancies (Hamid et al., 2015). For pancreatic cancer and malignant mesothelioma treatment, immunotherapy based on anti-CTLA-4, anti-PD-1, and anti-PD-L1 antibodies, has been tested with promising preliminary results (Le et al., 2013; Reck et al., 2013).

Another strategy to improve cancer vaccine efficacy could be the use of tumor antigen peptides. The most important contribute in the eradication of tumor is due to cytotoxix T cells (CTL), which recognized peptide loaded on MHC class I molecules on the cell surface. Identifying the specific peptides of a TAA that mark the tumor as dangerous, CTL can be activated and kill the tumor in a specific manner. Vaccines based on survivin multi-epitope peptides have been tested with positive results (Ciesielski et al., 2014; Widenmeyer et al., 2012; Miyazaki et al., 2011). Moreover, the combination of more TAAs shared by tumor seems to be a possible strategy (ClinicalTrials.gov NCT02239861; Bei and Scardino, 2010)

Next steps of the project could be: 1) to evaluate the intra-tumor infiltration of immune cells in order to evaluate the immune response induced by vaccination, 2) to evaluate the effect of the vaccination on the frequency of Survivin-positice cells, 3) to immunize mice with lipid-coated RNA to overcome all possible anti-vector responses and to increase the immune response using suitable formulation, 4) to combine self-amplifying RNA immunization with other immunotherapies, such as priming with Survivin-DNA, or boosting with Survivin protein, or with MVA encoding Survivin, or adding immune stimulating monoclonal antibodies (anti CTLA-4 or PD-L1). 4) To combine the RNA vaccine with other therapeutic strategies, such as chemotherapy. In the latter, the vaccine might allow the use of lighter chemotherapy protocols.

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